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Cloning and Characterization of a Novel Ion Channel

Jung-Ha Lee
Loyola University Chicago

LOYOLA UNIVERSITY OF CHICAGO

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Amino acids	A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cystine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; L, leucine; I, isoleucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine
AMV	avian myeloblastosis virus
BAPTA	bis-(o-aminophenoxy)ethane-N,N,N',N',-tetraacetic acid
Bay K 8644	1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid
BLAST	basic local alignment search tool
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfate
CIAP	calf intestine alkaline phosphatase
CMV	cytomegalovirus
cRNA	complementary ribonucleic acid
DEPC	diethylpyrocarbonate
DHP	1,4-dihydropyridine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
EC_{50}	half-maximal effective concentration
EDTA	(ethylenedinitrilo)tetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EST	expressed sequence tags
EtBr	ethidium bromide
FPL 64176	methyl 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1 H-pyrrole-3-carboxylate
GFP	green fluorescent protein
G protein	GTP binding protein
HEK293 cells	human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HVA	high voltage-activated
IC_{50}	half-maximal inhibitory concentration
I_{CRAC}	calcium release-activated calcium current
IP_3	inositol 1,4,5-triphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
LVA	low voltage-activated
MMLV	moloney murine leukemia virus
MOPS	4-morpholinepropanesulfonic acid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
Rb21-channel	a putative ion channel cloned from rat brain
RNase	ribonuclease
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate (or sodium lauryl sulfate)

TEA	triethyl ammonium
Tris	tri(hydroxymethyl)aminomethane
tsA201	human embryonic kidney cells transfected with the SV-40 large T-antigen
VACC	voltage-activated calcium channel
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

Electrical stimulation of voltage-activated calcium channels (VACCs) causes calcium entry in various excitable cells. Increase of cytoplasmic Ca^{2+} concentration is coupled with essential physiological functions including membrane depolarization, muscle contraction, hormone secretion, synaptic transmission, cell viability, and gene expression (Hille, 1992). Genetic defects of VACC subunits have been linked with several diseases such as: hypokalemic periodic paralysis, absence epilepsy, ataxia, and migraine (Lehmann-Horn and Rüdel, 1997).

Electrophysiological and pharmacological studies have identified how Ca^{2+} channels are related to pivotal physiological functions, and established the basic criteria for their classification. VACCs can be classified into low VACCs and high VACCs based on their different threshold for activation (Carbone and Lux, 1984). Low VACCs were characterized by their distinctive electrophysiological properties of low voltage threshold (-60 mV), transient time course, and about 8 pS of unitary conductance (Nilius *et al.*, 1985). High VACCs were characterized by their higher threshold for activation (-30 mV) than low VACCs. High VACCs can be further subclassified based on their pharmacology (table 1): (1) L-type Ca^{2+} channels (dihydropyridine-sensitive), (2) N-type Ca^{2+} channels (ω-conotoxin GVIA-sensitive), (3) P-type Ca^{2+} channels (ω-agatoxin IVA-sensitive), (4) Q-type Ca^{2+}

channels (MVIIC-sensitive), and (5) R-type Ca^{2+} channels (drug-resistant).

Biochemical purification of VACCs has shown that VACCs consist of $\alpha 1$, $\alpha 2/\delta$, and β subunits. Molecular cloning and expression studies have demonstrated that $\alpha 1$ subunits contain the channel pore and voltage sensor regions, which determine basic properties of VACCs, whereas $\alpha 2/\delta$ and β subunits are auxiliary subunits which regulate channel function (figure 1). According to expression studies on cloned Ca^{2+} channel $\alpha 1$ subunits, high VACCs can be further classified as follows: $\alpha 1S$ is a skeletal L-; $\alpha 1C$ is a cardiac L-; $\alpha 1D$ is a neuroendocrine L-; $\alpha 1A$ is a P/Q-; $\alpha 1B$ is a N-; and $\alpha 1E$ is a R-type Ca^{2+} channel $\alpha 1$ subunit (table 1; Birnbaumer *et al.*, 1994; Perez-Reyes and Schneider, 1994; Dolphin, 1995). Coexpression of the cloned $\alpha 1$ subunits with auxiliary subunits, $\alpha 2/\delta$, β, and γ, mimicked most of high VACC currents characterized from isolated cells electrophysiologically and pharmacologically.

T-type calcium channels are thought to be involved in important physiological functions such as pacemaker activity in cardiac and neuronal cells, smooth muscle contraction, hormone secretion, regulation of neuronal action potential, and some development. In spite of these essential roles, information about their structures, physiological roles, and regulation is lacking. Thus, cloning and expression studies of novel ion channels such as T-type Ca^{2+} channels would provide important clues to solve those basic questions.

The $\alpha 1$ subunit is a pore-forming protein composed of four homologous domains, each of which contains putative six membrane spanning segments and one pore loop (figure 1; Catterall, 1995). Significant structural similarity exists between a domain of VACC $\alpha 1$

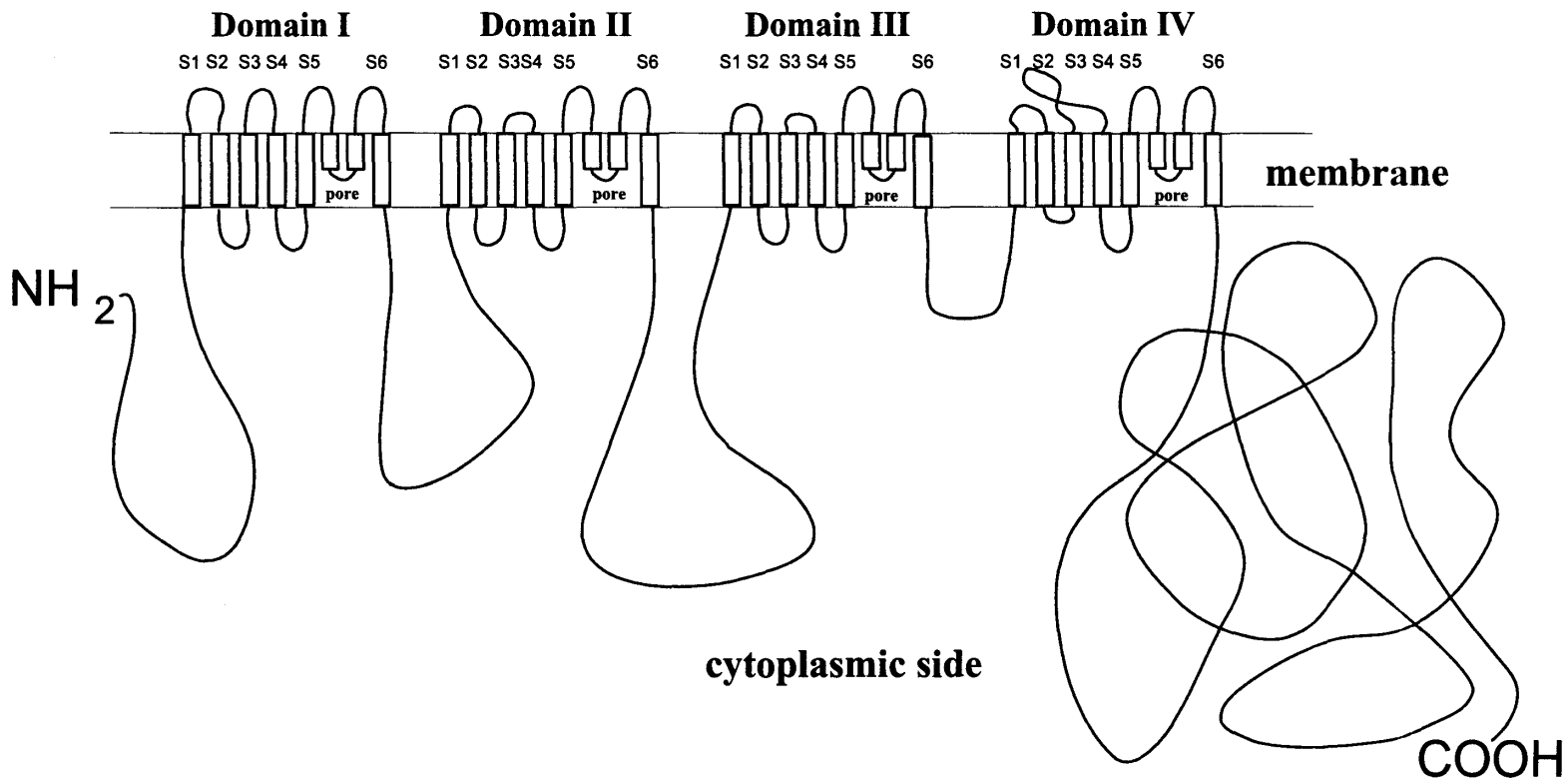

Figure 1. Structure of voltage-activated Ca^{2+} channel $\alpha 1$ subunits. The pore-forming subunit consists of four domains, each of which contains six membrane spanning portions (S1, S2, S3, S4, S5, and S6) and a pore loop. The S4 portions containing several positively-charged amino acids are known as voltage sensors .

Table 1. Assignment of cloned α1 subunits to functional Ca^{2+} channel types based on their characteristics.

Cloned Type	Dominant Expression	Functional Type	Threshold	Conductance	Antagonists
α1S	skeletal muscle	L-type	HVA	14, 20pS	DHP, diltiazem, verapamil
α1C	heart, lung, aorta, brain, fibroblast	L-type	HVA	24-25 pS	DHP, diltiazem, verapamil
α1D	brain, pancreas,	L-type	HVA		DHP
α1A	brain, kidney	P/Q-type	HVA	15-18 pS	ω-conotoxin MVIIC, sFTX, ω-agatoxin IVA
α1B	brain periperal neuron	N-type	HVA	14-16 pS	ω-conotoxin GVIA
α1E	brain, heart	R-type	HVA	13-14 pS	Resistant, Ni^{2+}
(?)	brain, heart, adrenal gland, skeletal muscle	T-type	LVA	7-9 pS	Ni^{2+}

HVA: High voltage-activated
DHP: Dihydropyridine
LVA: Low voltage-activated
sFTX: synthetic funnel web spider toxin

Contents of this table are adapted from the following papers: Flockerzi, *et al.*, 1986; Zhang *et al.*, 1993; Perez-Reyes and Schneider, 1994; Birnbaumer *et al.*, 1994; Bourinet *et al.*, 1996; and Dirksen *et al.*, 1995. Single channel conductances were measured with 100-120 mM Ba^{2+} as charge carrier. An α1 subunit for T-type Ca^{2+} channels has not been cloned yet.

and voltage-activated K^+ channel α subunits. A functional K^+ channel was found to have tetrameric stoichiometry (MacKinnon, 1991). Based on their structural homology in both gene and protein levels, an ancestral Ca^{2+} channel $\alpha 1$ subunit consisting of four domains was proposed to have resulted from two rounds of duplication of a single domain channel like a K^+ channel (Hille, 1992). From the evolutionary relationships of voltage-activated ion channels, it was assumed that low and high VACC $\alpha 1$ subunits might have diverged from a putative ancestral Ca^{2+} channel $\alpha 1$, and that conserved sequences between these two types might be found in Ca^{2+} channels of low-level and high-level living organisms.

In preliminary experiments, I applied reverse transcription-polymerase chain reaction (RT-PCR) cloning methods to clone a novel channel, such as a T-type Ca^{2+} channel. The initial strategy to design PCR primers was based on the hypothesis that novel Ca^{2+} channels contain consensus amino acid sequences found in mammalian high VACC sequences. PCR primers designed on conserved amino acids in reported mammalian $\alpha 1$ subunits should amplify not only already cloned $\alpha 1$ sequences, but also novel $\alpha 1$ sequences such as those of T-type channels. The PCR substrate was cDNA, which was reverse-transcribed from mRNA isolated from various tissues and cell lines that have been reported to express T-type Ca^{2+} channel currents. PCR products were separated, isolated, cloned, and sequenced. The deduced amino acid sequences of PCR products were compared to those of reported Ca^{2+} channel sequences. This comparison showed that a pair of PCR primers derived from mammalian Ca^{2+} channels amplified a putative Ca^{2+} channel cDNA fragment from *Paramecium tetraurelia*. However, the primers amplified only reported types of Ca^{2+} channel sequences from vertebrate tissues.

Another strategy to design PCR primers was based on conserved amino acid sequences found in less complex animal species. A Ca^{2+} channel-like sequence of simple animal species was searched for in the Genbank using BLAST (Basic Local Alignment Search Tool; Altschul *et al.*, 1990) program. A candidate for a novel Ca^{2+} channel sequence was found in *Caenorhabditis elegans* cosmid (C27f2). The deduced amino acid showed sequence homology to reported Ca^{2+} channels. Furthermore, the whole structure of the putative Ca^{2+} channel had four repeated domains, each of which contained six putative membrane spanning segments and a pore loop. To clone a rat version of the putative Ca^{2+} channel found in *C. elegans*, four pairs of PCR primers were designed on the basis of the *C. elegans* channel sequence. One pair of PCR primers amplified PCR products from rat brain. The translated sequence of a PCR product (designated Rb21) was homologous to reported Ca^{2+} and Na^{+} channel sequences, but was clearly different from those channels.

The working hypothesis was that the Rb21-PCR product was a partial cDNA sequence of a putative novel ion channel resembling Ca^{2+} and Na^{+} channels. This hypothesis was tested by the following specific aims. First, a rat brain cDNA library was screened to clone the full length cDNA of Rb21 (named as Rb21-channel). Second, Northern blot analyses were performed to localize the expression of Rb21-channel transcripts. Third, expression of the Rb21-channel was attempted in *Xenopus* oocytes and HEK 293 cells. Electrophysiological tools were applied to search for possible expression of the Rb21-channel.

CHAPTER II

LITERATURE REVIEW

2.1 Overview

Voltage-activated calcium channels (VACCs) have been extensively studied because they play pivotal roles in various physiological systems. The calcium ion gradient across the plasma membrane is so high that activation of VACCs by electric stimuli evokes external Ca^{2+} entry into the cytoplasm. Intracellular Ca^{2+} acts as a secondary messenger mediating diverse physiological functions such as: membrane depolarization in excitable cells, calcium-induced calcium release from sarcoplasmic reticulum in cardiac myocytes, exocytosis of vesicles containing neurotransmitters and hormones in nerve and endocrine cells, and even gene expression in most cells (Hille, 1992; Berne *et al.*, 1993; Ghosh and Greenberg, 1995).

Since the finding of Ca^{2+}-mediated action potentials in muscle fibers of crab legs, VACCs have been identified in all excitable cells ranging from single cell organisms such as *Chlamydomonas* (green alga) and *Paramecium* (protozoan), to vertebrates (Kung and Eckert, 1972; Harz and Hegemann, 1991; Hille, 1992). With development of voltage clamp and patch clamp techniques, the existence of multiple types of VACCs has been found from a variety of excitable cells. Multiple types were distinguished on the basis of biophysical and

pharmacological criteria (Tsien *et al.*, 1988; Bean, 1989). In cardiac cells, for example, low VACCs begin to activate around the resting membrane potential (-60 to -50 mV) and show a transient time course due to rapid inactivation (called T-type). T-type Ca^{2+} channels were further characterized by distinctive electrophysiological properties including slow deactivation time course, fast recovery from inactivation, and about 8 pS of unitary conductance (in 110 mM Ba^{2+} solution) (Hess, 1990; Huguenard, 1996).

On the contrary, high VACCs begin to activate around -30 mV and exhibit a long-lasting time course due to slow inactivation (Bean, 1985; Nilius et al., 1985; McDonald et al, 1994). Discoveries of selective organic blockers allowed high VACCs to be further classified into dihydropyridine (DHP)-sensitive L-type Ca^{2+} channels, ω-conotoxin GVIA-sensitive N-type Ca^{2+} channels, ω-agatoxin IVA-sensitive P-type Ca^{2+} channels, ω-conotoxin MVIIC-sensitive Q-type, and relatively drug-resistant R-type Ca^{2+} channels (Hess, 1990; Tsien and Tsien, 1990; Zhang *et al.*, 1993; Wheeler *et al.*, 1993).

Location and physiological functions of each high VACC were identified and re-evaluated using electrophysiological and pharmacological tools. DHP receptors in skeletal muscle are known to acts as voltage sensors as well as Ca^{2+} channels. Stimulation of the skeletal voltage sensors are coupled with Ca^{2+} release through ryanodine receptors from internal Ca^{2+} stores. The local Ca^{2+} increase by the initial Ca^{2+} release can cause additional Ca^{2+} release from the internal stores, inducing skeletal muscle contraction even without Ca^{2+} influx through the channel. On the contrary, cardiac muscle requires Ca^{2+} influx via the L-type channel to trigger Ca^{2+} release from internal Ca^{2+} stores (Bers, 1993). Apparently, movement of the S4 voltage sensors is not coupled to the cardiac isoform of the ryanodine

receptor. DHP-sensitive L-type Ca^{2+} channels identified in endocrine tissues such as adrenal chromaffin cells, pancreatic α and β cells, and pineal gland, trigger hormone secretion (Harrison *et al.*, 1989; Hsu *et al.*, 1991; Artalejo *et al.*, 1990, 1992; Gromada *et al.*, 1997). N-, P-, Q-, and R-type Ca^{2+} channels are coupled with neurotransmitter secretion in nerve terminals and hormone secretion in endocrine cells (Tsien *et al*, 1990; Hofmann *et al.*, 1994). Although multiple types of Ca^{2+} channels were identified from neuroendocrine cells, their contribution to cell depolarization, synaptic transmission, hormone secretion, and gene expression differs between cell types (Artalejo *et al.*, 1992; Randall *et al.*,1995; Wheeler *et al.*, 1995; Gromada *et al.*, 1997).

Biochemical purification and molecular cloning of Ca^{2+} channels have uncovered new aspects about structure, diversity, pharmacology, and biophysical properties of Ca^{2+} channels. The first Ca^{2+} channel was purified from skeletal muscle T-tubules using a dihydropyridine as marker. The partial amino acid sequence of the purified protein made it feasible to clone, and then to express the skeletal Ca^{2+} channel α1 subunit (Tanabe *et al.*, 1987 and 1988; Perez-Reyes *et al.*, 1989). Other Ca^{2+} channel α1 subunits were cloned by cDNA library screening using probes derived from either the cloned skeletal α1 cDNA, or PCR products. As summarized in table 1, up to now, six types of Ca^{2+} channels have been cloned and expressed in several expression systems. Biophysical and pharmacological properties of cloned Ca^{2+} channels were compared to those of Ca^{2+} channel currents (Birnbaumer *et al.*, 1994; Perez-Reyes and Schneider, 1994). Molecular biological tools using site-directed point mutations and chimeric constructs between different channels allowed characterization of roles of various portions in a cloned Ca^{2+} channel. Expression

studies of modified Ca^{2+} channels gave new insights to understanding how VACCs behave in terms of voltage-dependent activation and inactivation, drug-binding site, ion selectivity, and regulation (Perez-Reyes and Schneider, 1994; Dolphin, 1995).

In this chapter, it will be reviewed how high voltage-activated Ca^{2+} channels have been purified, cloned, and expressed in other systems. Since T-type Ca^{2+} channels remain to be cloned, biophysical characteristics and physiological functions of T-type Ca^{2+} channels will be discussed, thereby emphasizing the importance of molecular cloning of T-type Ca^{2+} channels. Finally, I will discuss what kinds of experimental methods could be applied to clone and characterize a novel ion channel such as a T-type Ca^{2+} channel.

2.2 Purification of Ca^{2+} channels

Ca^{2+} channels were first purified from skeletal muscle t-tubules where DHP (dihydropyridine) receptors are densely populated (50 pmol/mg protein, Fosset *et al.*, 1983). DHP derivatives, which were known as L-type channel specific blockers, have low dissociation constants to DHP-receptors, allowing them to be used as markers during purification. To isolate DHP-receptors, skeletal muscle t-tubules were first labeled with [^{3}H]-DHP and solubilized in detergents such as digitonin or CHAPS. Solubilized receptors were purified through wheat germ agglutinin chromatography, and ion exchange chromatography. Purified DHP-receptors were separated in SDS-polyacrylamide gels into $\alpha 1$ ($\alpha 1S$, 165-214 kDa), $\alpha 2\delta$ (170 kDa), β (55 kDa) and γ (32 kDa). The $\alpha 1$ (165-190 kDa) and $\alpha 2\delta$ (170 kDa) bands overlapped in gels due to their similar size. But $\alpha 2\delta$ could be separated in reducing conditions into $\alpha 2$ (142 kDa) and δ (25 kDa), which were distinguished

from α1 (Flockeri *et al.*, 1986a; Takahashi *et al.*, 1987; Tanabe *et al.*, 1987; Ellis *et al.*, 1988; Ruth *et al.*, 1989; Jay *et al.*, 1990; Bosse *et al.*, 1990; De Jongh *et al.*, 1991). Purified DHP-receptors showed Ca^{2+} channel activity in lipid bilayers, proving that DHP receptors are Ca^{2+} channels (Flockeri *et al*, 1986b).

Similar experimental approaches were used to purify cardiac L-type Ca^{2+} channels from several species including chick, cow, pig, and rat (Chang and Hosey, 1988; Schneider and Hofmann, 1988; Hasse *et al.*, 1991; Hell *et al.*, 1993). Using labeled-dihydropyridines as probes, cardiac L-type Ca^{2+} channel proteins were purified, showing at least 3 subunits, α1 (α1C), β, and α2/δ in SDS-polyacrylamide gels. The apparent molecular weight of purified α1C ranged from 165 to 235 kDa, suggesting that the diversity of protein sizes might be due to proteolysis or glycosylation or splicing variants. Such as skeletal DHP-receptors, the longer form of α1C containing C-terminal tail was preferentially phosphorylated by cAMP-dependent protein kinase. This result suggested that the cAMP-dependent protein phosphorylation site is in the C-terminal region (Chang and Hosey, 1988; Yoshida *et al.*, 1992; Hell *et al.*, 1993). The molecular weight of α2/δ was 170-190 kDa, which was divided into α2 (~150 kDa) and δ (~25 kDa) subunits in reducing conditions. The molecular weight of the β subunit was about 50 kDa (Chang and Hosey, 1988; Hasse *et al.*, 1991; Tokumaru *et al*, 1992).

ω-Conotoxin-GVIA, a toxin isolated from a marine snail (*Conus geographus*), irreversibly blocks N-type Ca^{2+} channel currents (Cruz and Olivera, 1986; Fox *et al.*, 1987a,b; Kasai and Neher, 1991). This pharmacological criterium established **N**-type Ca^{2+} channels (**N** for **N**either T nor L, found in **N**eurons) as a group of high VACCs different from

DHP-sensitive Ca^{2+} channels. Using ω-conotoxin-GVIA as marker, N-type Ca^{2+} channels (ω-conotoxin-GVIA receptors) were purified from brain tissues (McEnery *et al.*, 1991a; Witcher *et al.*, 1993). The purified ω-conotoxin-GVIA receptor complex consisted of α1 (α1B), β3, and α2/δ in SDS-polyacrylamide gels. Interestingly, these receptors were copurified with the following: an unidentified protein (95-100 kDa), synaptotagmin, syntaxin, and G protein α_o subunit (McEnery *et al.*, 1991b, 1992; Witcher *et al.* 1993; Lévêque *et al.* 1992, 1994). These results support the hypothesis that N-type channels interact with syntaxin and synaptotagmin, which were known to participate in synaptic vesicle docking and exocytosis. A recent study demonstrated that syntaxin could downregulate N- and Q-type Ca^{2+} channels by stabilizing inactivated states, which could be released by hyperpolarization (Bezprozvanny *et al.*, 1995). Accumulating evidence has shown that G-protein regulation of Ca^{2+} channel currents through membrane delimited pathways are diverse depending on multiple G-protein α (Dolphin, 1995) and βγ subunits (Ikeda, 1996). Protein interaction studies between various regions of Ca^{2+} channels and G protein subunits determined that the G-protein βγ binds on the loop between domain I and II of non-L-type Ca^{2+} channels (De Waard *et al.*, 1997; Zamponi, *et al.*, 1997).

Taken together, skeletal and cardiac L-type Ca^{2+} channels and neuronal N-type Ca^{2+} channels have been purified using specific ligands as probes. Distinct α1 subunits with their β and α2/δ auxiliary subunits were found in three Ca^{2+} channel complexes, while γ subunit was only detected in skeletal L-type Ca^{2+} channels. The expression of the γ transcript was detected in aorta and lung by Northerns (Jay *et al.*, 1990; Powers *et al.*, 1993).

2.3 Cloning and expression of Ca^{2+} channels

2.3a Cloning and expression of skeletal L-type Ca^{2+} channels (α1S)

The skeletal L-type Ca^{2+} channel was cloned by library screening using oligonucleotides derived from determined amino acid sequences of the purified DHP receptor. To clone subunits composing a skeletal DHP-receptor complex, each purified subunit was fragmented by trypsin treatment. Fragments were purified and partially sequenced by Edman degradation analysis. Degenerate oligonucleotides were synthesized on the basis of possible coding sequences of determined amino acid sequences. Oligonucleotides were used as probes to screen rabbit skeletal muscle cDNA libraries. Overlapping clones detected by probes were reassembled to obtain the full open reading frame using restriction enzyme sites. The open reading frame of the rabbit skeletal Ca^{2+} channel α1 subunit is 5,619 base pairs which encodes 1,873 amino acids. The molecular weight of the deduced α1 protein is 212 kDa (Tanabe *et al.*, 1987).

The overall amino acid sequence shared significant similarity with rat sodium channel II. Similarity matrix analysis showed that the DHP-receptor has four internal repeats (I, II, III, and IV) that share sequence homology one another. Hydropathy analysis showed that each repeat (also called domain) of the DHP-receptor has six membrane spanning segments (S1 to S6) plus a pore loop, which are also found in voltage-activated sodium channels (Noda *et al.*, 1984, 1986). These sequence and structural similarities suggested that DHP-receptors might be a VACC and the two channel families might have evolved from an ancestral type of voltage-activated ion channels consisting of four domains. Furthermore, comparison of the cloned Ca^{2+} channel with voltage-activated K^+ channels suggested that each domain of

the Ca^{2+} channel corresponds to the structure of most voltage-activated potassium channels (Jan and Jan, 1990). Especially, they share important structural and functional properties in voltage sensor portions (S4) determining channel activation. These facts suggested that the cloned Ca^{2+} channel is a member of voltage-activated ion channel superfamily containing Na^+ and K^+ channels.

Evidence that short segments (called SS1 and SS2) found between S5 and S6 of VACCs are involved in ion selectivity has come from site-directed mutation experiments of cloned Na^+ and Ca^{2+} channels (Heinemann *et al.*, 1992; Mikala *et al.*, 1993; Tang *et al.*, 1993; Yang *et al.*, 1993; Parent and Gopalakrishnan, 1995). Point mutations of the positively charged and neutral amino acids in pore loops of repeat III and IV of the Na^+ channel into negatively-charged amino acids changed Na^+ selectivity for the Na^+ channel to be permeable to divalent ions such as Ca^{2+} and Ba^{2+}. Similar point mutation experiments were performed in cloned Ca^{2+} channels where four glutamic acids are found in SS2 portions. Point mutations of each glutamate residue into neutral or positively-charged amino acids significantly changed Ca^{2+} ion selectivity and conductance (Mikala *et al.*, 1993; Tang *et al.*, 1993; Yang *et al.*, 1993; Parent and Gopalakrishnan, 1995). Voltage sensor portions (S4) contain several positively-charged amino acids (lysine or arginine) positioned in most every third position. Charge movement induced by structural changes of voltage sensors during electrical stimulation is considered to cause activation of voltage-activated ion channels (Stühmer *et al.*, 1989; Papazian *et al.*, 1991; Catterall, 1995; Yang *et al.*, 1995).

Auxiliary subunits of $\alpha 2$, β, γ, and δ, were cloned using similar methods applied to clone $\alpha 1S$ (Tanabe *et al.*, 1987; Ellis *et al.*, 1988; Ruth *et al.*, 1989; Jay *et al.*, 1990; Bosse

et at., 1990). Briefly, amino acids of purified accessory subunits were partially sequenced. Degenerate oligonucleotides were synthesized based on determined amino acid sequences, then used as probes to screen skeletal muscle cDNA libraries.

Skeletal L-type channel α1 was first expressed in dysgenic myotubules (Tanabe *et al.*, 1988) where slow Ca^{2+} channel current, charge movement, and excitation-contraction coupling were not detected (Beam *et al.*, 1986). Microinjection of expression vectors containing α1 cDNA caused not only expression of DHP-sensitive slowly activating Ca^{2+} channel currents, but also restoration of excitation-contraction coupling in dysgenic myocytes. Expression of skeletal L-type channel α1 in dysgenic myotubules also supported that skeletal L-type channel α1 subunits act as voltage sensors. In absence of external Ca^{2+} source, excitation of expressed skeletal L-type channels increased of cytoplasmic Ca^{2+} by Ca^{2+} release from internal Ca^{2+} stores, resulting in muscle contraction (Ríos and Brum, 1987; Tanabe *et al.*, 1990a,b).

Skeletal Ca^{2+} channel α1 subunit was also expressed in stably-transfected mouse L-cells where endogenous Ca^{2+} channel current or an α2 subunit was not detected (Perez-Reyes *et al.*, 1989). The measured current was stimulated by (-) Bay K 8644, a DHP agonist of L-type channels. However, the activation of the expressed current was 1000-fold slower than that of the current measured *in vivo*. These results revealed that α1 alone was able to evoke functional L-type currents in terms of DHP-sensitivity and voltage-activated ion channel. Also, abnormally slow activation by α1 alone suggested that there might be important modulatory functions of auxiliary subunits. Later, the coexpression of α1 with β subunit caused normalization of current activation kinetics and increase of DHP-binding sites

(Lacerda *et al.*, 1991). Recently, Ren and Hall expressed the rabbit skeletal L-type Ca^{2+} channel α1S with β1b in *Xenopus* oocytes (1997). They found that the β1b subunit (Pragnell *et al.*, 1991) is necessary to express the α1S subunit, and that the α2δ subunit significantly increases the expressed current in *Xenopus* oocytes.

2.3b Cloning and expression of cardiac L-type Ca^{2+} channels (α1C)

Since the cloning of skeletal muscle Ca^{2+} channel α1 (α1S) subunit using channel purification, other kinds of Ca^{2+} channel α1 subunits have been cloned by the following methods. First, homologous Ca^{2+} channel sequences were cloned by screening cDNA libraries with α1S cDNA sequences as probes under low stringency conditions (formed by increased salt concentration or decreased washing temperature). Second, similar Ca^{2+} channel cDNA fragments could be amplified by polymerase chain reaction (PCR) for which primers were designed based on consensus sequences of cloned Ca^{2+} channel sequences.

The cardiac L-type Ca^{2+} channel α1 (α1C) cDNA was cloned from rabbit heart cDNA library by screening under low stringency conditions with skeletal L-type Ca^{2+} channel cDNA sequences as probes (Mikami *et al.*, 1989). The full length open reading frame was 6,513 nucleotides encoding 2,171 amino acids of which the estimated molecular mass was 242,771 Da. The cardiac L-type Ca^{2+} channel revealed 66 % sequence identity with the rabbit skeletal L-type Ca^{2+} channel α1. Amino acid sequences in voltage sensor regions (S4) and pore loops were well conserved between the two channels. Hydropathy analysis suggested that each of four domains consists of six hydrophobic segments plus a pore loop. These results suggested that the cloned α1C is a homologue of skeletal α1S. Northern blot analysis, PCR, and *in situ*

hybridization revealed that cardiac L-type channels transcripts are expressed in various tissues, including heart, brain, lung, smooth muscle, trachea, and ovary (Mikami *et al.*, 1989; Schultz *et al.*, 1993; Perez-Reyes *et al.*, 1990). Cardiac L-type mRNA was also detected in adrenal glomerulosa, and *Xenopus* oocytes by PCR (see Section 4.1).

Mikami and his colleagues (1989) first expressed functional cardiac L-type Ca^{2+} channels by injecting *Xenopus* oocytes with *in vitro* transcribed cRNA of α1C. Injected oocytes showed inward Ba^{2+} currents, which were stimulated by 5 μM Bay K 8644, but inhibited by 1 μM nifedipine. They also showed that coexpression of the cardiac L-type Ca^{2+} channel α1 with skeletal α2 caused an increase of current amplitude without changing the peak current-voltage relationship and DHP-sensitivity. However, later studies in mammalian cells found that α2 subunits increase DHP binding sites and DHP-sensitivity (Wei *et al.*, 1995). Coexpression of α1C with β subunits produced an increase of peak current, a negative shift of the peak current-voltage relationship, increase of DHP-sensitivity and binding sites, and a modulation of activation and inactivation time constants (Wei *et al.*, 1991, 1995; Perez-Reyes *et al.*, 1992, 1994).

Activation of cardiac L-type Ca^{2+} channels causes Ca^{2+} entry across the membrane due to Ca^{2+} gradient between extracellular and cytoplasmic sides. Increased cytoplasmic Ca^{2+} concentration induces Ca^{2+} release from sarcoplasmic reticulum, resulting in cardiac muscle contraction. In contrast to skeletal L-type Ca^{2+} channels, which act as voltage sensors rather than Ca^{2+} channels, Ca^{2+} influx through cardiac L-type Ca^{2+} channels is necessary to induce cardiac muscle contraction (Bers, 1993). In dysgenic myotubules, expression studies of skeletal and cardiac L-type Ca^{2+} channels confirmed that the difference between cardiac- and

skeletal-type muscle contraction is basically due to the distinguished roles of the two DHP-receptors (Tanabe *et al.*, 1990a, b). Expressed cardiac L-type channels in myotubules required entry of external Ca^{2+} for excitation-contraction, while expressed skeletal channels did not require external Ca^{2+} for excitation-contraction. Using chimeras constructed between these two cloned channels, the loop between repeat II and III of the skeletal Ca^{2+} channel was identified as a portion which might physically contact with the Ca^{2+} release channel (called ryanodine receptor) in sarcoplasmic reticulum.

L-type Ca^{2+} channels in heart can be regulated by protein kinase A-dependent phosphorylation which is mediated by β-adrenergic receptor stimulation (McDonald *et al.*, 1994). Purification of cardiac DHP-receptors revealed size heterogeneity which might be due to C-terminal truncated forms of α1C (Chang and Hosey, 1988; Schneider and Hofmann, 1988; Hasse *et al.*, 1991; Hell *et al.*, 1993). Only the long size was cAMP-dependently phosphorylated (Yoshida *et al.*, 1992). These reports suggested that the full-length form and the C-terminal truncated forms are differently expressed and regulated. Wei and his colleagues showed that C-terminal deletion mutants of α1C significantly enhanced current amplitude without modifying gating charges, suggesting that the current was increased by facilitating the coupling between the charge movement and the channel opening (Wei *et al.*, 1994). Similar to the effect of deletion of cardiac L-type channel terminal, trypsin treatment through patch pipettes increased current amplitude (Hescheler and Trautwein, 1988). Recently, the cAMP-dependent protein kinase A phosphorylation site in the C-terminal end of the cardiac L-type Ca^{2+} channel was determined by point mutation experiments (Gao *et al.*, 1997). They showed that regulation of cardiac L-type Ca^{2+} channel by protein kinase A

requires an A-kinase anchoring protein called AKAP79 (Coghlan *et al.*, 1995; Faux and Scott, 1996). One of the possible interpretations is that the C-terminal of cardiac L-type Ca^{2+} channels may usually inhibit channel activity, but phosphorylation may eliminate the inhibition by the C-terminal tail via a conformational change. Another possible interpretation is that α1C may interact with β subunits differently depending on its phosphorylation state.

2.3c Cloning and expression of neuroendocrine L-type Ca^{2+} channels (α1D)

The neuroendocrine L-type Ca^{2+} channel (α1D) was cloned from a rat brain cDNA library by screening in low stringency conditions using the skeletal L-type α1 cDNA sequence as probe (Hui *et al.*, 1991). Structurally the channel has four repeats, each of which contains six hydrophobic portions and a pore loop. The translated sequences of α1D showed 71 % and 76 % homology to cardiac and skeletal L-type Ca^{2+} channel α1 subunits. These facts suggested that this channel is another L-type Ca^{2+} channel whose properties might be similar to previously cloned L-type channels. However, the deduced amino acid sequence had a shorter C-terminal tail compared with those of cardiac and skeletal L-type Ca^{2+} channels. The α1D isoform cloned from hamster HIT cells also had an early stop codon in the C-terminal tail like the rat version of α1D. However, a human α1D cDNA cloned from a cDNA library of a human neuroblastoma IMR 32 cell line revealed a long C-terminal tail isoform (Williams *et al.*, 1992b). Recently, Ihara and his colleagues cloned a splice variant from rat insulinoma (RINm5F) cDNA library which showed a long C-terminal tail such as the human version α1D (Ihara *et al.*, 1995). Combination of the different size α1D splicing

variants and their possible auxiliary subunits implies diversity of neuroendocrine L-type Ca^{2+} channels.

Northern blot analyses and PCR detected α1D-transcripts in brain, pancreas, kidney, ovary, heart, and adrenal glomerulosa cells (Perez-Reyes *et al.*, 1990; Seino *et al.*, 1992; Yaney *et al.*, 1992; refer to Section 4.1). Additionally, Northern blot anlysis using the 3'-untranslated region as probe showed that α1D-transcript expression could be detected in brain, pancreas, heart, and skeletal muscle (Yaney *et al.*, 1992). Recently, an antibody developed from the exofacial pore loop in domain IV of α1D significantly blocked cardiac Ca^{2+} channel currents, suggesting that cardiac myocytes express α1D-Ca^{2+} channels as well as α1C-Ca^{2+} channels (Wyatt *et al.*, 1997). The blocking behavior by this antibody supports the structural and functional model of ion permeation through a pore in VACCs. Furthermore, the classic notion that α1C is the only Ca^{2+} channel in heart should be modified. However, additional experiments should be required before accepting the modified notion. For example, whether the antibody is specific or not should be reexamined in expressed currents of cloned Ca^{2+} channels.

The human version of α1D was first expressed in *Xenopus* oocytes with auxiliary subunits, α2 and β2 (Williams *et al.*, 1992b). The measured current was stimulated by an L-type Ca^{2+} channel agonist, Bay K 8644, but inhibited by a L-type Ca^{2+} channel antagonist, nifedipine. Interestingly, the expressed current showed reversible blockade to 10 μM ω-conotoxin GVIA which is a N-type Ca^{2+} channel blocker. However, native Ca^{2+} channel currents stimulated by injection of an auxiliary β_2 subunit could be bigger than the α1D expressed currents (Lacerda *et al.*, 1994; Perez-Reyes and Schneider, 1994). Moreover, the

β2-amplified endogenous current was blocked by low concentrations of ω-conotoxin GVIA. These results suggested that the expressed currents of human α1D might be contaminated by native oocyte currents stimulated by α2 and β2 subunits. Recently, we observed that injection of α2 and β2 subunits into *Xenopus* oocytes was able to induce L-type Ca^{2+} channel currents which were stimulated by (-)Bay K 8644 or FPL 64176 treatment (Lee and Perez-Reyes, unpublished data). Both splice variants of the rat α1D were expressed in CHO cells with β2 (Ihara *et al.*, 1995). The expressed currents were 30-80 pA in 40 mM Ba^{2+} solution and stimulated by (±) Bay K 8644 treatment. However, it cannot be ruled out that Bay K 8644-stimulated currents might originate from β2-stimulated CHO-endogenous currents because the measured current densities were very small. Thus, the biophysical and pharmacological properties of cloned α1D remains in question due to the lack of robust expression.

2.2d Cloning and expression of neuronal P/Q-type Ca^{2+} channels (α1A)

The first α1A cDNA was cloned from rabbit brain by screening a cDNA library with the skeletal L-type Ca^{2+} channel α1 cDNA as probe (Mori *et al.*, 1991). Two splice variants having different C-terminal ends were detected. The two deduced proteins consist of 2,273 (253,337 Da) and 2,424 (273,217 Da) amino acids. The deduced α1A protein shared similar structure with previously cloned skeletal and cardiac L-type Ca^{2+} channel α1 subunits in terms of four domain structure and the hydropathy profile of each domain containing six putative membrane spanning portions and a pore loop. The amino acid sequence homology of the α1A to the previously cloned three L-type α1 subunits was 42-44 %. This relatively

low sequence homology suggested that α1A might pertain to a subfamily different from L-type α1 subunits (α1S, α1C and α1D). The corresponding membrane spanning portions revealed high homology, but the connecting loops were less conserved.

Northern blot and PCR analyses detected α1A-transcripts in many brain tissues including cerebellar Purkinje and granular cells, hippocampus, olfactory bulb, and spinal cord (Mori *et al.*, 1991; Starr *et al.*, 1991). They were also detected from kidney cortex, heart, *Xenopus* oocytes, and adrenal glomerulosa (Mori *et al.*, 1991; Yu *et al.*, 1992; see Section 4.1).

Injection of *in vitro* synthesized α1A-cRNA into *Xenopus* oocytes evoked a high voltage-activated Ca^{2+} channel current that was not blocked by either nifedipine (L-type Ca^{2+} channel specific blocker) or ω-conotoxin GVIA (N-type Ca^{2+} channel blocker), but by crude venom from *Agelenopsis aperta* (Mori *et al.*, 1991).

Coexpression of α1A with the skeletal α2 or β1 increased the current amplitude by about 3- or 20-fold, while the coexpression with both auxiliary subunits caused a 200-fold enhancement of the current amplitude. These results suggested that the brain α1A subunit might be associated with α2 and β1 subunits, such as the skeletal L-type DHP receptor. Also, the coexpression with both of the skeletal α2 and β had synergistic effects on the functional α1A Ca^{2+} channel expression. The single channel conductance was 16.5 pS in 110 Ba^{2+} (Mori *et al.*, 1991). Initially, the cloned α1A was assumed to correspond to a P-type Ca^{2+} channel found in cerebellar Purkinje neurons. However, considerable differences exist between the expressed current of α1A and a P-type Ca^{2+} channel current in terms of current kinetics and pharmacology. P-type currents measured from cerebellar Purkinje neurons were

characterized by their noninactivating current kinetics and high sensitivity to ω-agatoxin IVA (IC_{50} = 2 nM) (Llinas *et al.*, 1992; Mintz *et al.*, 1992). However, the expressed current of α1A showed inactivating kinetics and was less sensitive to ω-agatoxin IVA (IC_{50} = 200 nM) (Sather *et al.*, 1993), suggesting that α1A might be a novel class of high voltage-activated Ca^{2+} channels. Another pharmacological criterium to separate α1A-expressed currents from P-type currents is that α1A-expressed currents are 10 fold more sensitive to block by the cone snail toxin, ω-conotoxin MVIIC than P-type channel currents (IC_{50}<150 nM for α1A currents expressed in oocytes; IC_{50}>1 μM for P-type currents) (Sather, *et al.*, 1993; Wheeler *et al.*, 1994; Randall and Tsien, 1995). In spite of these pharmacological and electrophysiological differences, an α1 subunit mimicking P-type Ca^{2+} channel current has not been cloned yet.

Reviewing the cloning of sodium channels showed that there are at least 9 types of voltage activated Na^+ channels. Atypical Na^+ channels (Na2.1 and Na2.3) share only 40-52 % sequence homology with the other 8 types of Na^+ channels (George *et al.*, 1992; Felipe *et al.*, 1994). Diversity of cloned Na^+ channels implies another possibility that the P-type α1 gene might be so different from already cloned Ca^{2+} channel sequences that the cDNA was not cloned by general cloning methods including library screening in low stringency conditions and PCR using primers derived from conserved amino acid sequences in Ca^{2+} channel sequences. Another explanation is that the P-type α1 gene might be α1A, and that the reported differences could be caused by splice variants of α1A and/or diverse combination of auxiliary subunits.

2.3e Cloning and expression of N-type Ca^{2+} channels (α1B)

The α1B cDNAs were cloned from rat brain and human IMR32 neuroblastoma cDNA libraries simultaneously by the same method of screening the libraries in low stringency conditions using the skeletal L-type α1S cDNA as probe (Dubel *et al.*, 1992; Williams *et al.*, 1992a). Subsequently, the rabbit α1B was cloned from a rabbit brain cDNA library using a rabbit α1A fragment as probe (Fujita *et al.*, 1993). The translated α1B sequence showed 43-51 % identity to three L-type Ca^{2+} channel α1 subunits, while the sequence showed 82 % and 78 % identity to α1A and α1E. According to an evolutionary tree based on amino acid sequence identity, the α1B Ca^{2+} channel was classified into a non-L-type subfamily distinguished from the L-type subfamily (Fujita *et al.*, 1993).

From Northern blot analyses and PCR, the α1B transcript was detected in brain, but not in skeletal muscle, aorta, heart, testis, kidney, spleen, liver, lung, or adrenal gland (Williams *et al.*, 1992a; Dubel *et al.*, 1992; Fujita *et al.*, 1993).

Human α1B was coexpressed with neuronal α2 and β2 subunits in mammalian HEK293 cells by transient transfection (Williams *et al.*, 1992a). Expression of the human α1B was detected as inward currents. Pharmacologically, the measured current was irreversibly blocked by ω-conotoxin GVIA (IC_{50}=55 pM), but insensitive to dihydropyridines. Binding assays using ^{125}I-ω-conotoxin GVIA showed that α1Bβ2α2-transfected cells had ten times higher B_{max} than α1B- or α1Bα2-transfected cells. Coexpression studies of α1B with the auxiliary subunits found that a β was necessary to express normal N-type Ca^{2+} activity, while the α2 subunit tended to increase the expression of the channel. In *Xenopus* oocytes, the modulatory roles of β1 on α1B were detected with

increases of expressed currents, altered activation and inactivation kinetics, and a shift of voltage-dependent inactivation (Stea *et al.*, 1993). α1B was also expressed in dysgenic myotubules. The single channel conductance was 15 pS in 110 mM Ba^{2+} solution (Fujita *et al.*, 1993). Reconstitution of the purified receptors in lipid bilayers showed functional Ca^{2+} channel activity (De Waard *et al.*, 1994).

2.3f Cloning and expression of R-type Ca^{2+} channels (α1E)

The first α1E cDNA was cloned from a rabbit brain cDNA library using α1A as probe (Niidome *et al.*, 1992). Sequence homology analyses suggested that α1E was a member of the non-L-type Ca^{2+} channel subfamily including α1A and α1B, since the α1E sequence shared higher similarity with α1A (59 %) than the L-type Ca^{2+} channel α1 subunits (42 %). Other α1E subunits from rat, human, and marine ray were cloned by RT-PCR plus subsequent cDNA library screening (Ellinor *et al.*, 1993; Horne *et al.*, 1993; Soong *et al.*, 1993; Schneider *et al.*, 1994).

Northern blot analysis and *in situ* hybridization showed that α1E transcripts were mainly detected in the central nervous system. In brain, the signals were dominantly detected in cerebral cortex, corpus striatum, cerebellum, and hippocampus (Niidome *et al.*, 1992; Soong *et al.*, 1993). Additionally, α1E mRNA was detected in *Xenopus* oocytes, rat heart, and bovine adrenal glomerulosa cells by PCR (refer to Section 4.1).

Nuclear injection of the vector containing rat α1E cDNA in *Xenopus* oocytes induced fast activating and inactivating currents (Soong *et al.*, 1993). Interestingly, expressed currents began to activate at a test pulse of -40 mV from a holding potential of -100 mV and

showed a peak current at -10 mV in 4 mM Ba^{2+} solution. Compared to previous cloned high voltage-activated Ca^{2+} channels including L-, N-, P/Q-types, the α1E-expressed currents began to activate at more hyperpolarized potentials and decayed faster during prolonged test pulses. Based on these properties, they suggested that α1E could be a low voltage-activated Ca^{2+} channel (Soong *et al.*, 1993). However, the other α1E subunits cloned from marine ray, mouse, rabbit, and human evoked currents whose thresholds and peak current-voltage relationships pertain to characteristics of high VACCs (Ellinor *et al.*, 1993; Schneider *et al.*, 1994; Wakamori *et al.*,1994; Williams, *et al.*, 1994). Single channel conductances of cloned α1E subunits were about 14 pS (Ellinor *et al.*, 1993; Zhang *et al.*, 1993; Schneider *et al.*, 1994), while those of T-type channels were about 7-9 pS in 100-115 mM Ba^{2+} (Huguenard, 1996). Furthermore, T-type channels have lower threshold (-60 to -50 mV) and distinctively slower deactivation kinetics than α1E-channels (Carbone and Lux, 1984 and 1987; Tsien *et al.*, 1988; Randall and Tsien, 1997).

Pharmacologically, the α1E-expressed currents were not affected by known organic blockers including dihydropyridines, ω-conotoxins, and FTX (Ellinor *et al.*, 1993; Soong *et al.*, 1993; Zhang *et al.*, 1993; Schneider *et al.*, 1994). Because the α1E-expressed currents were resistant to organic drugs, α1E-channels are called R-type channels (Zhang *et al.*, 1993). Recently, Randall and Tsien (1997) demonstrated that T-type currents in undifferentiated NG108-15 have distinguished biophysical and pharmacological properties from drug-resistant R-type currents in cerebellar granular cells as well as α1E-expressed currents. They concluded that drug-resistant currents (R-type currents) share similar biophysical and pharmacological properties with α1E-expressed currents.

2.4 T-type Ca^{2+} channels

Expression studies of six types of Ca^{2+} channel α1 subunits with their auxiliary subunits mimic most properties of Ca^{2+} channel currents measured from most isolated cells except T-type Ca^{2+} channel currents. Thus, T-type Ca^{2+} channels are the only subgroup of characterized VACCs remaining to be cloned.

Despite their smaller amplitude than high-threshold Ca^{2+} currents, T-type Ca^{2+} currents have been found in a variety of tissues, such as: cardiac muscle, smooth muscle, developing skeletal muscle, fibroblasts, osteoblasts, astrocytes, neurons, certain developing cells, adrenal glomerulosa cells and other secretory cells (Bean, 1989). The diverse distribution of T-type Ca^{2+} channels suggests that they participate in a wide variety of physiological functions, including cardiac pacemaking activity (Bean, 1985; Hagiwara *et al.*, 1988), smooth muscle contraction (Ganitkevich *et al.*, 1990), hormone secretion (McCarthy *et al.*, 1993; Mlinar *et al.* 1993), neuronal activity (Suzuki *et al.*, 1989), and some developmental roles (Xu *et al.*, 1990).

Existence of multiple T-type Ca^{2+} channels has been proposed from distinctive biophysical and pharmacological profiles of T-type Ca^{2+} channel currents (Akaike *et al.*, 1989; Huguenard, 1996). T-type Ca^{2+} channel currents found in some neuronal cells, adrenal glomerulosa cells, and sperm cells, were blocked by DHPs, especially nicardipine, while other T-type currents were insensitive to DHPs (Cohen *et al.*, 1988; Akaike *et al.*, 1989; Richard *et al.*, 1991). Diverse pharmacological profiles of T-type currents was summarized by Huguenard (1996). Recently, Tarasenko and his colleagues (1997) described that two subtypes of low VACCs exist in thalamocortical neurons based on current kinetics and

pharmacology. One subtype dominantly detected at the earliest postnatal stage showed relatively fast decaying time course and was blocked by La^{3+} and nifedipine. The other subtype detected in at least two weeks old cells was characterized by relatively slow decaying time course and Ni^{2+}-sensitive blockade.

In contrast to high VACCs, T-type channels can be characterized by their distinctive electrophysiological properties including more negative voltage activation, transient time course, slower deactivation in tail currents, and small unitary conductance (~8 pS in 110 mM Ba^{2+} solution) (Chen and Hess, 1990). In fact, the single channel conductance of T-type channels for Ca^{2+} ions is similar to those of high voltage-activated Ca^{2+} channels (Bourinet *et al*, 1996). Ca^{2+} ions can pass more efficiently through T-type channels than high VACCs, because only low VACCs are activated by small depolarizations of the resting membrane potential where there is a greater driving force for Ca^{2+} (Hille, 1992; Huguenard, 1996). Consequently, T-type channels may mediate the initiation and regulation of the cardiac pacemaker potential, hormone secretion, and neuronal action potentials.

In spite of T-type channels being involved in important physiological functions, their structures and regulations are lacking. Therefore, it would be very important to clone novel ion channels such as T-type Ca^{2+} channels in order to show structural differences from other ion channels. The expression of cloned ion channels in other simple systems may provide us insights to understand the following questions. What are the physiological functions of the cloned channels? How are the channels regulated? How are the biophysical properties of the channels coupled with their structure? Also, T-type Ca^{2+} channels may be purified using antibodies developed against specific sequences of cloned channels. Purification of

native T-type Ca^{2+} channels may allow us to understand the native structure of T-type channels. Furthermore, expression of cloned ion channels in other systems could contribute to development of new drugs if newly cloned ion channels are coupled with a specific disease.

2.5 Possible experimental approaches to clone a T-type Ca^{2+} channel

One of the methods to clone a channel cDNA is via protein purification, as was done for skeletal L-type Ca^{2+} channels, but there is no specific blocker which can be used as a marker for T-type channel purification. Even though several candidates for T-type Ca^{2+} channel blockers have been proposed such as Ni^{2+}, phenytoin, tetramethrin, niguldipine, and U-92032 (Bean, 1989; Xu and Lee, 1994), their blocking behaviors overlapped between different Ca^{2+} channels. Moreover, these blockers were not specific enough to be used as probes for purification of T-type Ca^{2+} channels. Compared to cloning via purification, cloning of Ca^{2+} channels via RT-PCR might be a method which could overcome problems such as a small population of channels and a lack of specific blockers.

Recently, a consensus interaction site of β subunits on the domain I to II loop of α subunits was identified by screening a library containing various parts of the α1S subunit with labelled β protein (Pragnell *et al.*, 1994; De Waard *et al.*, 1995). This information provides a possible approach to purify a novel α1 such as a T-type using the consensus interaction site of β subunits as a specific ligand. However, it is still controversial whether β subunits cloned from high voltage-activated Ca^{2+} channels also modulate T-type Ca^{2+} channels or not (Lacerda *et al.*, 1994; Lambert *et al.*, 1997).

Another possible approach to clone a T-type channel gene is expression cloning, which has been used successfully to clone several novel potassium channels (Frech *et al.*, 1989; Ho *et al*, 1993). The first step of T-type channel cloning by this method would be to check whether low VACC currents can be expressed from *Xenopus* oocytes after injection of poly(A)$^+$ RNA isolated from tissues expressing T-type currents. If expression of T-type currents can be detected from the oocytes, the second step would be to identify which size of mRNA can express T-type currents. A cDNA library can be synthesized from the mRNA of the identified size. Repeated expression studies of cRNAs made from continual fractionations of the cDNA library could be performed to purify a clone encoding T-type channels.

Only one study has found that low VACC currents can be expressed in *Xenopus* oocytes by injection of poly(A)$^+$ RNA isolated from rat thalamohypothalamic regions (Dzhura *et al.*, 1996). Measured Ba^{2+} currents were activated at -70 mV, but were not transient currents. However, most other studies showed that mRNAs of rat heart and brain from which T-type currents have been identified did not induce any T-type current activity in *Xenopus* oocytes (Leonard *et al.*, 1987; Lory *et al.*, 1990). The first step to check whether expression cloning can be used to clone a T-type channel was performed as a preliminary experiment. However, T-type currents were not detected from oocytes injected with total RNA or poly(A)$^+$ RNA of NIE-115 neuroblastoma cells, which were reported to predominantly express T-type currents (Liévano *et al.*, 1994). These results suggested that the functional assay method might be not applied to clone a T-type channel cDNA.

T-type Ca^{2+} channels share common properties with other VACCs in terms of

selectivity for Ca^{2+} ions, voltage-dependent activation, and some dihydropyridine sensitive blockade. T-type Ca^{2+} channels are selectively permeable to Ca^{2+} ions, which is similar to properties of high VACCs. Another similar property is that T-type channels are activated by depolarizing voltages, even if they have a lower threshold. Pharmacologically, T-type Ca^{2+} currents found in neuronal cells and adrenal glomerulosa cells were blocked by nicardipine, an L-type Ca^{2+} channel antagonist (Cohen *et al.*, 1988; Akaike *et al.*, 1989; Richard *et al.*, 1991). The activity of T-type current in adrenal glomerulosa cells was modulated by intracellular phosphorylation pathways (Lu *et al.*, 1994). These common properties provided the background for my working hypothesis that amino acid sequences of T-type Ca^{2+} channels may be similar with the amino acid sequences of already cloned Ca^{2+} channels, specially in pore loop and voltage sensor portions. If so, PCR primers designed on the basis of conserved amino acids in cloned Ca^{2+} channels might amplify T-type Ca^{2+} channel cDNAs as well as the cloned Ca^{2+} channel cDNAs.

Another alternative cloning method is to find novel channel sequences by searching the Genbank where numerous unidentified sequences are deposited. Using the BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool; Altschul *et al.*, 1990) program of the National Center for Biotechnology Information, we can search for interesting cDNA or protein sequences. For example, the human *Trp* gene fragment (Genbank access number; EST05093) was found in the Genbank database using a homologous sequence of the *Drosophila trp* (Zhu *et al.*, 1995). To get a full-length cDNA of the human *Trp*, a human cDNA library was then screened by a synthetic oligonuotide sequence corresponding to the human *Trp* gene fragment. The partial cDNA sequence (Genbank access number; Z45660) of a novel non-

voltage-activated sodium channel (BNC1 for Brain Na^+ Channel) was found in the Genbank using a similar process (Price *et al.*, 1996). They searched the database of expressed sequence tags (EST) in the Genbank using the BLAST program for which the amino acid sequences of previously cloned degenerins and ENaC (amiloride-sensitive epithelial Na^{2+} channel) (Waldmann *et al.*, 1995; Benos *et al.*, 1997).

Reviewing the cloning history of VACCs suggested that PCR cloning was a powerful tool to clone novel channels that could be used when direct purification and expression cloning of T-type channels are not available. A primitive Ca^{2+} channel-like sequence was found in the Genbank database using the BLAST program. In this study, a putative ion channel (Rb21-channel) from rat brain was cloned by the combined approaches of Genbank search and PCR cloning, followed by cDNA library screening. Structural analyses of the Rb21-channel suggested that the putative ion channel is a member of the 4 domain cation channel superfamily such as voltage-activated Ca^{2+} and Na^+ channels.

CHAPTER III

METHODS

3.1 PCR cloning

Reverse transcription-polymerase chain reaction (RT-PCR) was applied to clone novel Ca^{2+} channel sequences from various tissues that have been reported to express T-type Ca^{2+} channels. Degenerate PCR primers were designed based on consensus sequences of reported Ca^{2+} channels. The designed PCR primers were summarized in Table 2. PCR substrate was reverse transcribed from total RNAs or poly(A)$^+$ RNAs isolated from *Xenopus* oocytes, bovine adrenal glomerulosa cells, rat heart, rat brain, *Paramecium tetraurelia*, and NIE-115 neuroblastoma cells. Amplified PCR products were cloned, sequenced, and translated into amino acid sequences. The deduced sequences of PCR products were identified by comparing the sequences with known Ca^{2+} and Na^+ channels.

3.1a Design of PCR primers

Structural and functional studies of voltage-activated K^+ channels demonstrated that a functional channel requires an assembly of 4 α subunits, each of which contains 6 membrane spanning segments plus a pore loop (MacKinnon, 1991; Schulteis *et al.*, 1996). Structural comparison of K^+ channels to voltage-activated Ca^{2+} and Na^+ channels comprising

four domains showed that one K^+ channel subunit corresponds to one domain of Ca^{2+} and Na^+ channel α subunits. Based on their structural analogy, Hille (1992) proposed that Ca^{2+} channel $\alpha 1$ subunits consisting of 4 domains resulted from double duplication of an original domain during the long evolutionary period. Amino acid sequence comparison between domains showed that domain I is most homologous to domain III, while domain II is most similar to domain IV. Consensus portions in Ca^{2+} channel domain I and III, which were presumed to be similar to the original domain, were found from amino acid sequence alignment of reported Ca^{2+} channel $\alpha 1$ subunits.

The first PCR primers were designed from the consensus amino acid sequences in putative membrane spanning segment 3 (S3) and pore regions of domain I and III, GWN(V/L)(L/M)D and TMEGW(T/P). The DNA sequences encoding these amino acids were predicted from their usage codons. Because most amino acids are encoded by multiple codons, the code is called degenerate. For example, valine is encoded by the 4 triple nucleotide sequences, GTA, GTT, GTC, and GTG. The sequences of PCR primers were as follows: forward primers (C8), TCCTTAA GGC TGG AA(C/T) (G/C)TN (A/C)TN GA(C/T) TT, and reverse primers (C9),CGGATC CGT CCA NCC (C/T)TC CAT NGT. All sequences were written from the 5' to 3' direction. N represents four kinds of nucleotides and other alternative forms of nucleotides are expressed within the parenthesis. All primers were synthesized at the Macromolecular Analysis Facility (Loyola University Medical Center).

L-type specific PCR primers were designed on the basis of conserved amino acid sequences found in putative membrane spanning segment 6 (S6) in repeat III and IV of

Primer	Direction	Location	Amino acid sequence	Annealing Temp.	Predicted size
C8	forward	DIS3	GWN(V/L)(L/M)D	58°C	500 bp
C9	reverse	DIpore	TMEGW(T/P)	58°C	
C3	forward	DIIIS6	FMMNIFV	60°C	950 bp
C4	reverse	DIVS6	FISFYMLC	60°C	
T1	forward	DIIS5	GMQLFG	51°C	114 bp
T2	reverse	DIIpore	VFQILTGE	51°C	
T3	forward	DIIIS3	RN(Y/L)WNILD	57°C	
T4	reverse	DIIIS5	VQLFKGK	57°C	280 bp
T5	reverse	DIIIpore	T(F/G)EGWP	57°C	470 bp
T6	forward	DIVS3	DAWN(I/V)FDF	54°C	260 bp
T7	reverse	DIVS5	GMQ(V/M)FG	54°C	
T11	forward	DIS5	MGVQ(L/I)FG	53°C	275 bp
T12	reverse	DIpore	EGWVYV(L/I)YD	53°C	
T13	forward	DIIIS5	GVQLVGGK	54°C	245 bp
T14	reverse	DIIIpore	FETLSYKG	54°C	
T15	forward	DIVS5	AGVVLFG	50°C	128 bp
T16	reverse	DIVpore	TGEDWNDI	50°C	

Table 2. Summary of PCR primers. Name, direction, location, and corresponding amino acid sequence of each PCR primer and optimal annealing temperatures of PCR reactions were described. Location of each primer represents where the corresponding amino acid sequence is in reported Ca^{2+} channel sequences or the putative *C. elegans* ion channel sequence in cosmid C27f2. For example, DIS3 represents the putative membrane spanning segment 3 (S3) of domain I (DI). T11 to T16 were designed on the basis of the translated sequence of C27f2, while the other primers were designed based on reported mammalian Ca^{2+} channel sequences. Using the Oligo program (National Biosciences, Inc.), annealing temperature (Ta) was calculated from following formula: Ta = 0.3 x Tm (primer) + 0.7 x Tm (product) - 25.

published L-type Ca^{2+} channel $\alpha 1$ subunits, FMMNIFV and FISFYMLC. Possible DNA sequences encoding these amino acids were predicted from their degenerate codons. Sequences of PCR primers were as follows: the forward primer (C5), CGCGGAATTC TT(T/C) ATG ATG AA(C/T) AT(T/C/A) TT(T/C) GT, and the reverse (C6), CGGCGGAT CCA NAG CAT (A/G)TA (A/G)AA (A/G)CT (A/G/T)AT (G/A)AA.

Additionally, three pairs of PCR primers were derived from reported Ca^{2+} channels and the putative *C. elegans* Ca^{2+} channel sequence in a cosmid C27f2. T15 and T16 primers, which amplified a rat version cDNA fragment of the putative *C. elegans* channel sequence, were designed based on amino acid sequences in a putative membrane spanning segment 5 (S5) and a pore of the domain IV, AGVVLFG and TGEDWNDI. Corresponding DNA sequences encoding these amino acids were from their usage codons. Complementary DNA sequences of PCR primers were as follows: the forward primer (T15), GCC GGN GTN GTN (C/T)TN TT(C/T) GG, and the reverse primer (T16), AT (A/G)TC (A/G)TT CCA (A/G)TC(C/T)TC NCC NGT.

3.1b Purification of RNAs

Total RNAs were purified from *Xenopus* oocytes, bovine adrenal glomerulosa cells, rat heart, rat brain, *Paramecium tetraurelia*, and NIE-115 neuroblastoma cells using guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). For example, 0.5 to 1 gram of tissue or 10^7 to 10^8 cells were ground with a glass homogenizer or a polytron in 5 ml of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7, 0.5 % sarcosyl, and 0.1 M 2-mercaptoethanol). The homogenate was mixed

sequentially with 0.5 ml of 2 M sodium acetate, pH 4, 5 ml of phenol, and 1 ml of chloroform. The final mixture was shaken vigorously and incubated on ice for 10 min, followed by centrifugation at 10,000 xg for 20 min. The upper aqueous phase containing RNA was transferred to a new tube, and then RNA was precipitated with 5 ml isopropanol at -20°C for 1 hr. The RNA was collected by centrifugation at 10,000 xg for 20 min. The precipitate was washed with 70 % ethanol and dried in air. The dried RNA was dissolved in RNase-free H_2O, which was prepared by diethyl-pyrocarbonate (DEP)-treatment and autoclaving. The quality and quantity of isolated RNA was estimated spectrophotometrically. Usually 1 to 2 mg of RNA was obtained from 1 gram of tissue or 10^8 cells, and the ratio of O. D. values measured at 260 and 280 nm was about 1.8. If the ratio of O. D. values was lower than 1.8, isolated total RNA was further purified by repeating the described procedure. Rat heart and brain RNAs were isolated from fetal Sprague-Dawley rats. In the case of oocyte RNA, poly(A)$^+$ RNA was purified from total RNA using a mini-oligo(dT)$^+$ cellulose column kit (5 prime→3 Prime, Inc or Boehringer Mannheim) (Sambrook *et al.*, 1989). The yield of poly(A)$^+$ RNA purification from total RNA was about 1 % and the 260/280 nm ratio of O. D. values was 1.9.

3.1c Synthesis of first strand cDNA and polymerase chain reaction (PCR)

First strand cDNA was synthesized from 0.2 to 1 μg of total RNA or poly(A)$^+$ RNA using 20 units of AMV (**a**vian **m**yeloblastosis **v**irus) reverse transcriptase (Boehringer Mannheim) or MMLV (**m**oloney **m**urine **l**eukemia **v**irus) reverse transcriptase (Gibco BRL), 4 μl of 5X reaction buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 100-

200 pM reverse primers or random primers, 1 mM dNTPs, and 50 units of RNase inhibitor, in a final volume of 20 µl. After mixing and centrifuging, the reaction mixture was incubated at room temperature for 10 min and then at 42°C for 60 min, followed by 95°C for 5 min to terminate the reaction. Reverse-transcribed cDNAs were used as substrates for PCR. The first strand cDNA reaction was supplemented with 2.5 units of Taq polymerase (Perkin Elmer), 100 pM of each upstream and downstream primer, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and H_2O to the final volume of 100 µl. PCR reaction was carried out in an Ericomp thermocycler. The specific annealing temperatures for PCR primers were displayed in Table 2. For C8 and C9 primers, two different annealing temperatures were used. After the first cycle (30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C) was repeated 15 times, then the second cycle (30 sec at 94°C, 30 sec at 65°C, and 30 sec at 72°C) was repeated 20 times. PCR products were separated through a 0.8 % agarose gel containing 1 µg/ml of ethidium bromide by electrophoresis. PCR products of the expected size were cut out of the gel and purified with glass powder (GeneClean) or Qiaquick gel extraction columns (Qiagen).

3.1d Cloning of PCR products

Taq DNA polymerase synthesizes cDNA products based on DNA templates, and adds a single deoxyadenosine (A) to the 3'-end of PCR products, which is a non-template-dependent activity. Thus, PCR products containing additional 3'-A can be efficiently subcloned into pGEM-T (Promega) or PCR 2.1 vectors (Invitrogen), which were prepared by adding a deoxythymidine to both 3' ends of vectors after blunt-ended digestion. The

ligation was performed in a final volume of 10 µl containing 1 unit of T4 ligase (Promega or Pharmacia), 25 ng PCR product, 50 ng vector, 3 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 1 mM DTT, and 0.05 mM ATP. The ligation reaction was incubated at 15°C for 3-4 hours or 12°C overnight. The reaction was finished by heating at 72°C for 3-5 min, followed by slow cooling to room temperature. Two or three µl of the ligation mixture was used to transform JM109 *E. coli* frozen competent cells (Sambrook *et al.*, 1989). Transformation was carried out by incubating on ice for 20-30 min, at 42°C for 90 sec, and on ice for 2 min, followed by adding of 500 µl of SOC (20 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 40 mM glucose) to the reaction, followed by incubation at 37°C for 1 hour for recovery. Cells transformed by pGEM-T vectors not containing PCR products become not only ampicillin-resistant, but also β-galactosidase producible. On the contrary, cells transformed by pGEM-T vectors containing PCR products become ampicillin-resistant, but can not produce β-galactosidase. The production of β-galactosidase can be detected in presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), a histochemical substrate, which is changed into blue precipitates by the enzyme. To select colonies whose plasmids contain PCR products, transformation mixture was plated on agar plates containing 50 µg/ml ampicillin, treated with 50 µl of 25 % X-gal, and 50 µl of 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG). White colonies growing on the agar plates were cultured in 3-5 ml of SOC medium containing 50 µg/ml of ampicillin at 37°C. Plasmid DNA was isolated from each culture using the alkaline lysis method (Sambrook *et al.*, 1989). Plasmid DNA was digested with restriction enzymes that cut polylinkers adjacent to an insert and separated on 1% agarose gels containing 1 µg/ml of

ethidium bromide to check insertion of a PCR product in the vector.

3.1e Sequencing, translation, and identification of PCR products

Plasmids containing PCR products were further purified by polyethylene glycol precipitation. Precipitated plasmids were collected by centrifugation at 10,000 xg for 5 min at 4°C. Each pellet was washed with 70 % ethanol, recentrifuged, dried in air, and then dissolved in 30 µl H_2O. Each plasmid DNA was denatured with 3 µl of 2 M NaOH for 3 min at room temperature, then precipitated with 4.5 µl of 3 M ammonium acetate and 105 µl of 95 % ethanol. Each denatured plasmid was resuspended in 10 µl of Sequenase buffer (40 mM Tris-Cl, pH 7.5; 20 mM $MgCl_2$; and 50 mM DTT) containing 1-2 pM M13 reverse primer or T7 forward primer. Primers were annealed to denatured plasmids by heating at 65°C for 2 min and then cooling slowly to room temperature for 20-30 min. The principle of sequencing by the dideoxy-termination method (Sanger *et al*, 1977) is based on the random termination of DNA polymerization by incorporation of dideoxynucleotides, which lack the 3'-OH group necessary for DNA elongation. The first step of this procedure is the DNA polymerase reaction to elongate sequences from the annealed primers in the presence of dNTPs plus labeled deoxynucleotides. During the DNA polymerase reaction, labeled deoxynucleotides will incorporate into elongating DNAs. Aliquots of the reaction were then transferred to different tubes containing each dideoxynucleotide, resulting in random termination of DNA synthesis by the competitive incorporation of dideoxynucleotides. Different lengths of DNA sequences can be separated by electrophoresis and detected by autoradiography of the gel. Briefly, DNA polymerization was performed by adding 5.5 µl

of master mixture (1 µl of 0.1 M DTT, 2 µM of dNTPs, 0.5 µl of [^{35}S]-dATP, and 2 µl of Sequenase) to primer-annealed plasmids. The reaction was incubated at room temperature for 3 to 5 min and then 3.5 µl of the reaction was transferred to each termination tube (dideoxy A, C, G, and T), followed by incubation of the termination reactions at 37°C for 5 min. The reactions were terminated by inactivating the DNA polymerase. Sequencing products were separated by electrophoresis through 6.5 % acrylamide (19:1, acrylamide: bis-acrylamide) gel containing 8 M urea in 1.2 X TBE (12 mM Tris, 10.6 mM boric acid, and 0.3 mM EDTA, pH 8.3). One X TBE (10 mM Tris, 8.9 mM boric acid, and 0.25 mM EDTA, pH 8.3) was used as the running buffer for electrophoresis. The gel was dried at 80°C for 1 hr under vacuum and then exposed to film for 10 - 15 hours. Nucleotide sequences were read from the autoradiograph using a digitizer, and then translated into amino acid sequences using a WDNASIS software (Hitachi). Translated sequences were manually compared with reported Ca^{2+} channel α1 sequences. Unidentified sequences not similar to known Ca^{2+} channels were compared with reported sequences deposited in Hitachi CD-DATA disks using a WDNASIS program.

3.2 Screening a rat brain cDNA library

A putative Ca^{2+} channel of *C. elegans* homologous to high VACC channels was found in the Genbank database as a homologous sequence to high VACC α1 subunit sequences. A rat version (Rb21) of the putative *C. elegans* Ca^{2+} channel sequence was cloned by RT-PCR cloning. The deduced amino acid sequence of the Rb21 cDNA fragment showed significant homology to reported Ca^{2+} channel sequences (figure 8). To clone the

full length cDNA of Rb21, a rat brain library was screened with the Rb21 cDNA fragment as probe. The whole library screening process including library plating, immobilization on nylon membranes, and hybridization was performed according to the manufacturer's protocol (Clontech).

3.2a Characteristics of the rat brain library

A rat brain cDNA library was purchased from Clontech (catalog number; RL3005a). The library was prepared based on Gubler and Hoffman procedure (1983). The first strand DNA was synthesized from poly(A)$^+$ RNA isolated from whole brain of adult male Sprague-Dawley rats (*Rattus norvegicus*) using oligo (dT) plus random primers. After synthesizing the second strand cDNA and adding *Eco* RI adapters, the double strand cDNA was ligated into λgt10 vectors which were digested by *Eco* RI and dephosphorylated by calf intestinal alkaline phosphatase (CIAP). Independent clones were about 2 million. The average insert size was 1.7 kbases. The library was amplified once. The titer was greater than 10^8 plaque forming unit (pfu)/ml.

3.2b Library plating and immobilization on nylon membranes

Approximately 0.6 million plaques were plated on 12 plates. Plaques were transferred on nylon membranes. Their DNAs were denatured and immobilized on membranes for hybridization.

First, the plaque forming unit (pfu)/ml was determined by titering the library. C600 *E. coli* host cells were cultured in 10 ml of LB broth (10 g/L Bacto-tryptone, 5 g/L Bacto-

yeast extract, and 5 g/L NaCl) containing 10 mM $MgSO_4$ and 0.2 % maltose at 37°C overnight. Density of host cells were adjusted to an O. D. of 0.5 at 600 nm with fresh LB broth. A series of library dilutions were incubated with 200 µl of C600 host cells at 37°C in a water bath for 15 min. Three ml of 48-50°C LB top agar (LB plus 0.7 % agarose) was added to each mixture and poured on a 100 mm LB agar plate. After cooling at room temperature for 10 min, plates were incubated at a 37°C for 6-7 hours. Titer (pfu/ml) was determined by multiplying following factors: number of plaques formed/µl used, dilution factors, and 10^3 µl/ml. Based on the calculated pfu/ml, 50,000 pfu/plate were plated on 12 LB agar plates (150 mm) so that the total plaques screened were about 0.6 million. These plates were incubated at a 37°C for 6-7 hours to allow plaques to grow to about 0.5 mm in size. These plates were then chilled at 4°C overnight to stop growth of plaques and to prevent the top agar from sticking to nylon membranes. Using forceps, a nylon membrane (Hybond-N; Amersham) was placed onto each plate, allowing plaques to transfer for 2 min. An 18 gauge needle was pricked through the membrane and the agar plate to mark orientation. To make duplicate nylon membranes, a second membrane was placed on the plate, and plaques were allowed to transfer for 4 min. The plaques and their DNAs on the nylon membrane were denatured by submerging the membrane in the denaturation solution (1.5 M NaCl and 0.5 M NaOH) for 2 min. The membrane was neutralized by submerging the membrane in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) solution. The membrane was rinsed in 0.2 M Tris-HCl (pH 7.5) and 2X SSC buffer solution (0.3 M NaCl, 30 mM sodium citrate, pH 7.0 adjusted with NaOH). After blotting on paper and drying at 37°C for 30 min, the membrane was baked at 80°C for 2 hours to crosslink the λ DNA to the membrane.

3.2c Hybridization and isolation of positive clones

To decrease non-selective hybridization, each membrane was prehybridized for 3-6 hours at 42°C in the following solution: 6X SSC (0.9 M NaCl, 90 mM sodium citrate, pH 7.0 adjusted with NaOH), 50 % formamide, 5X Denhardt's reagent (0.5 g Ficoll, 0.5 g polyvinylpyrrolidone, 0.5 g bovine serum albumin), 0.5 % sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA.

For the first probe, Rb21 cDNA was labeled using the RadPrime DNA labeling system (Gibco BRL). Briefly, Rb21 PCR products were digested out of vectors and separated on 0.8 % agarose gels. The expected size band corresponding to Rb21 was cut and purified through a Qiaquick gel extraction column (Qiagen). Purified cDNA was denatured by heating at 99°C for 5 min, followed by immediate cooling on ice. T15 and T16 specific primers were annealed to the denatured DNA and extended by 5 units of Klenow fragment of DNA polymerase I in the presence of 1 μM α-^{32}P-dCTP (3000 Ci/mmole), 5 μM dATP, 5 μM dTTP, and 5 μM dGTP in a final volume of 50 μl. The reaction was performed at 37°C for 5-15 min and stopped by adding 5 μl of 0.2 M EDTA. To estimate the incorporation of ^{32}P-dCTP into probes, 1 μl of the mixture was first diluted with 499 μl TE buffer (10 mM Tris-HCl , pH 8; 1 mM EDTA). Two equal volume aliquots of the dilution were spotted on separate glass fiber filters. One of the filters was washed three times to remove unincorporated ^{32}P-dCTP with 20 ml of cold 10 % (w/v) trichloroacetic acid containing 1 % sodium pyrophosphate and then one time with 95 % ethanol. The washed filter was dried at room temperature for 20 min. The other unwashed filter was used to determine the total radioactivity of ^{32}P-dCTP in the reaction. The radioactivities of washed and unwashed filters

were counted in the presence of 5 ml of scintillation cocktail (Fisher) with an LS 600SC scintillation counter (Beckman). The calculated radioactivity of the probe was 40 million cpm (counts per minute). Percent incorporation was 40 % and specific activity was 36000 Ci/mmole.

Before hybridization, the used prehybridization solution was replaced with fresh prewarmed solution. Prepared ^{32}P-labeled probes were denatured by heating at 99°C for 5 min and then added to the solution. Hybridization was performed at 42°C for 12-16 hours.

Membranes were washed from low to high stringency conditions by increasing temperature and decreasing salt concentration. The first wash was in a 2X SSC and 0.1 % SDS solution at room temperature for 10-15 min with shaking. The second wash was in fresh solution at 40°C for 20 min. After changing the solution again, washing was continued at 50°C for 15 min with shaking. At 50°C, the washing solution was switched with a 0.5X SSC and 0.1 % SDS solution and washing was performed for 20 min. The final washing was in a 0.1X SSC and 0.1 % SDS for 20 min at 50-55°C. During the washing process, relative radioactivities of washing solution and membranes were intermittently checked by a Geiger counter. After blotting on papers and drying at 37°C for 30 min, the membranes were exposed to X-ray films (Kodak, X-OMAT). These films (Kodak) were kept at -80°C for 24 to 48 hours in cassettes with two intensifying screens and then developed. In contrast to nonspecific bindings, specific bindings of probes would be found at the same positions on duplicate films. Overlapped positions were assumed to contain positive clones containing homologous or identical sequences to Rb21 cDNA. To isolate putative positive plaques, agar pieces under the black spots on the films were cut out with Pasteur pipettes and

transferred into 1 ml of SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 20 mM Tris-HCl, pH 7.5) and 20 µl of chloroform. Phages were eluted into the SM buffer by vortexing and incubating for 3 hours at room temperature. The eluted solution was titered with C600 host cells on LB agar plates. For the secondary screening, about 500 plaques were plated on a LB agar plate (100 mm). Plaque lifts were performed from pre-chilled plates. Membranes were denatured, neutralized, and washed in the solutions as before. Membranes were dried and baked at 80°C for 2 hours to cross-link λ DNAs on membranes. DNAs on membranes were hybridized, washed, and exposed to X-ray films by the same procedures explained above. Tertiary or quaternary screening was performed until all the plaques on a plate gave positive signals.

3.2d Identification of insert sizes of positive clones

To identify the size of each insert in positive clones, inserts were amplified by PCR using primers derived from the flanking portions of the *Eco* RI cloning site. Sizes of PCR products separated on a gel were compared to size markers. The forward primer is 5'AGCAAGTTCAGCCTGGTTAAGT3' and the reverse primer is 3'GGGACCTTCTTTAT-GAGTATT5'.

Agar pieces containing plaques were placed into 250 µl of distilled water and briefly vortexed. Phage DNA was released out of its capsule by repeating the cycle of freezing and thawing 2-3 times. The eluted solution containing DNA was heated at 94°C for 5 min to inactivate protease. A master mixture containing 1.5 unit of Taq polymerase (Perkin Elmer or Pharmacia), 100 pM of each primer, 5 µl of 10X PCR buffer (10 mM Tris and 50 mM

KCl; pH 8.3), 0.2 mM dNTPs, and 1.5 mM $MgCl_2$, was added to the prepared λ DNAs to a total volume of 50 μl. Each PCR reaction was carried out in an Ericomp thermocycler programmed to repeat the following 3 different cycles. The first cycle (40 sec at 94°C, 30 sec at 50°C, and 1 min at 72°C) was repeated 5 times. The second cycle (20 sec at 94°C, 20 sec at 50°C, and 1 min 20 sec) was repeated 15 times. The third cycle (20 sec at 94°C, 20 sec at 50°C, and 2 min) was repeated 10 times. PCR products were separated alongside kbase-size markers (Gibco BRL) in a 1 % agarose gel containing 1 μg/ml of ethidium bromide. Comparison of the PCR products to the size markers showed the size of each positive clone. Among the positive clones detected by library screenings, clones having big inserts were selected for the following experiments including isolation, subcloning, and sequencing.

3.2e Isolation, subcloning, and sequencing of DNAs in positive clones

To characterize positive clones more conveniently, λ DNAs were purified from isolated positive λ clones and then their inserts were digested, and subcloned into bacterial vectors. Subcloned inserts were sequenced in both directions with primer walking.

The λ DNAs containing big inserts (>1.5 Kb) determined by PCR were purified according to the following procedure. First, C600 *E. coli* host cells were cultured in 10 ml of LB broth (10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, and 5 g/L NaCl) containing 10 mM 10 mM $MgSO_4$ and 0.2 % maltose at 37°C overnight. Next, each isolated positive λ phage was titered with dilutions of 10X and 100X. On the basis of the titer, 10^4 pfu of each clone were plated on 5 plates (100-mm) containing LB broth, agarose (15 g/L), and 10

mM $MgSO_4$. The plaques were allowed to grow to confluence. Ten ml of λ dilution buffer (0.1 M NaCl, 0.01 M $MgSO_4$, 0.035 M Tris-HCl (pH 7.5), and 2 % gelatin) was added to each plate, and then incubated at 4°C overnight. After adding 10 µl of chloroform to each plate, plates were swirled briefly. The volume of collected lysate was adjusted to 50 ml with λ dilution buffer, followed by addition of 50 µl of chloroform. The plate lysate was centrifuged at 5000xg for 30 min to remove bacterial debris and residual agarose. The supernatant containing λ phages was collected into a new tube. The λ DNAs were isolated using the lamda medi kit (Qiagen). Briefly, bacterial DNA and RNA in lysates were digested by DNase I and RNase A. The λ phages were precipitated by addition of 10 ml of 30 % polyethylene glycol 600 and 3 M NaCl. Precipitated phages were harvested by centrifugation at 10000xg for 10 min. The supernatant was discarded and the pellet was resuspended. The resuspended phage particles were lysed by incubating at 70°C in 2 % sodium dodecyl sulfate solution. Lamda capsules were precipitated by adding 9 ml of the 3 M potassium acetate. The turbid solution was centrifuged at 15,000xg for 30 min and the particle free lysate was obtained from the supernatant. The λ DNA in the clean supernatant was purified using a lambda midi column (Qiagen). The amount of purified DNA was quantified spectrophotometrically.

The insert in the purified λ DNA was subcloned into the pGEM-3Z vector (Promega). Inserts in the λ vectors were digested by *Eco* RI, separated in an agarose gel, and purified using a gel extraction column (Qiagen). The pGEM-3Z cloning vector (Promega) was prepared as follows. First, the pGEM-3Z plasmid was digested by *Eco* RI and then the enzyme was inactivated by heating at 65°C for 20 min. Second, to prevent self-ligation, the

digested pGEM-3Z vector was dephosphorylated by the treatment of 0.1 unit of calf intestinal alkaline phosphatase (CIAP) at 37°C for 20-30 min. The dephosphorylation reaction was stopped by heating at 85°C for 15 min. Digested and dephosphorylated vectors were purified by phenol/chloroform extraction and ethanol precipitation. Purified lambda inserts were subcloned into prepared vectors by ligation using 1 unit of T4 ligase (Promega), 1 µl of 10 X reaction buffer (30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, and 0.5 mM ATP), 20-50 ng of inserts, and 30-100 ng of vectors in a final volume of 10 µl. The ligation reaction was incubated at 14°C for 3-4 hours and then heated at 72°C for 3 min, followed by cooling to room temperature. Two or three µl of ligation mixture was used to transform INFα *E. coli* competent cells (Invitrogen). Transformation was performed by incubating on ice for 30 min, at 42°C for 30 sec, then return to ice for 2 min. Five hundred µl of SOC was added to the transformation reaction. Transformants were shaken at 37°C for 1 hour, then plated on LB plates containing 50 µg/ml ampicillin. Colonies grown on the ampicillin LB plates were cultured in a small scale (3-5 ml) to check whether the plasmid isolated from each colony contains the same size of insert digested out from the positive lambda DNA. For large scale preparations of plasmid DNA, 250 ml of LB containing 1.25 mg ampicillin was inoculated with 3-5 ml of the precultured bacteria. Plasmids were purified from large scale bacterial cultures using plasmid maxi prep kits (Qiagen). Inserts subcloned in pGEM-3Z vectors were sequenced by the dideoxy-termination method (Sanger *et al.*, 1977). The 5' and 3' ends of inserts were sequenced using T7-forward and M13-reverse primers whose corresponding sequences are in the upper and lower portions adjacent to the cloning site. Translated sequences were compared with those of the putative *C.*

elegans and reported Ca^{2+} channels to determine the relative positions in the whole construct of Rb21-channel. Additional sequencing primers were designed based on end portions of sequences obtained using T7 and M13 primers. For the first positive clone, sequencing was performed until forward direction sequencing overlapped with reverse direction sequencing. For other clones, sequencing was continued until overlapping the previously obtained sequence.

Library screening was repeated three times to isolate positive clones covering the entire coding region of the putative ion channel (named as Rb21-channel). Positive clones containing relatively big inserts (>3 kbases) were subcloned and used to construct the full-length cDNA. Relative position and size of the probes used for library screening and the positive clones used for the full length cDNA construct were summarized in figure 10. Briefly, the first probe, Rb21 PCR product (4054 - 4181), was used to isolate the clone lam20 (3542 - 6555), which encodes from domain IV to the untranslated region. The second probe, the 5' end of lam20 digested by *Eco* RI (polylinker) and *Bam* HI(3703), was used to isolate d39 (2043 - 6720) and g2 (1372 - 3630). The d39 clone encodes from the untranslated 3' end up to the II-III loop. The g2 clone goes from the middle portion of domain II to domain IV. The third probe, the 5' end of g2 digested by *Eco* RI (polylinker) and *Hind* III (1522), was used to isolate rbIII7 and f2 clones. The rbIII7 (-100 - 1414) encodes from the 5' untranslated portion to the middle of domain II. The f2 clone (264 - 2037) encodes domain I S2 to the II-III loop.

3.2f Construction of the full-length cDNA of Rb21-channel in pGEM-3Z and pTracer

The initial full-length cDNA of Rb21-channel was obtained from the following overlapping clones; rbIII7, g2, and d39. The 5' portion of Rb21-channel was prepared by digesting rbIII7 clone with *Eco* RI (-64) and *Xma* I (1505). The middle portion was obtained by digesting the g2 clone by *Xma* I (1505) and *Nco* I (3394). The 3' portion was prepared by digesting the d39 clone by *Nco* I (3394) and *Sac* I (polylinker). The vector was prepared by digesting pGEM-3Z by *Eco* RI and *Sac* I. The three fragments and the digested vector were separated on an agarose gel and purified through gel purification columns (Qiagen).

The three purified fragments were ligated into the prepared vector in one reaction by using 1 unit of T4 ligase in presence of 1 µl of 10X reaction buffer (30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, and 0.5 mM ATP) in a final volume of 10 µl. The ligation reaction and the subsequent transformation were performed as before (Section 3.2e). Colonies grown on the ampicillin LB plates were cultured in a small scale (3 ml). Plasmids isolated from grown colonies were digested by *Eco* RI to check which plasmids contain a full length cDNA of the Rb21 channel. Candidates containing the full construct were further characterized by other restriction enzymes including *Xma* I and *Nco* I. Full-length clone candidates were further checked by sequencing. Ligated portions of 5' (*Eco* RI, -64) and 3' ends (*Sac* I in polylinker) and other ligated portions (*Xma* I, 1505; *Nco* I,3394) were sequenced by the dideoxy-termination method (Sanger *et al.*, 1977). Because a deletion of the domain IIS6 portion was found in the initial full-length construct, the missing portion was replaced with a *Xma* I (1505) and *Eco* RI (2037) digested fragment of f2. Later the full-length cDNA of Rb21-channel cloned in pGEM-3Z vector was sequenced in both directions to confirm whether there were errors in the cDNA construct. To express the Rb21-channel

in a mammalian expression system such as HEK 293 cells, the full-length cDNA was subcloned into pTracer (Invitrogen), a mammalian expression vector that has a CMV (cytomegalovirus) promoter and the gene for a green fluorescent protein.

3.3 Northern blot analyses

The tissue expression of the Rb21-channel transcript was investigated by Northern blot analyses. Total RNA isolated from rat tissues was separated on an denaturing gel, then transferred and immobilized on a nylon membrane. The size and relative expression level of Rb21-channel transcripts was examined by hybridizing the immobilized RNA on a membrane with labeled Rb21 cDNA. Hybridizations were also performed using a human multiple tissue blot and a human brain tissue blot purchased from Clontech.

3.3a Preparation of a rat multiple tissue blot

Total RNA was purified from lung, heart, skeletal muscle, testis, brain, kidney, and liver of a young male Sprague-Dawley rat (200 g) using guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The procedure was described previously (Section 3.1b). Quantities of the isolated RNAs were measured spectrophotometrically. Ratios of O. D. values between 260 and 280 nm were 1.7-1.8. A denaturing agarose gel was prepared as follows. Fifty five ml of H_2O containing 0.75 g of agarose was boiled and then cooled to 60°C, followed by addition of 7.5 ml of 10X MOPS buffer (0.2 M 4-morpholinopropansulfonic acid, 82 mM sodium acetate, 10 mM EDTA, pH 7.0) and 12.3 ml of 37 % formaldehyde. Twenty µg of each RNA sample was mixed with

a denaturing solution to become 6.5 % formaldehyde, 50 % formamide, 0.1 % EtBr, and 1 X MOPS buffer in a final volume of 20 µl. The mixture was heated at 72°C for 5-10 min before loading on the gel. The RNA was separated in the denaturing gel surrounded by 1 X MOPS running buffer (0.02 M 4-morpholinopropansulfonic acid, 8.2 mM CH_3CO_2Na, 1 mM EDTA, pH 7.0) by electrophoresis (at 100 mV for 2-3 hours). RNAs and size markers separated on the gel were visualized by UV illumination and photographed alongside a fluorescent ruler. Sizes of the RNA markers were plotted against the distance migrated. This semilogarithmic curve was used to calculate sizes of transcripts detected by hybridization.

Formaldehyde in the gel was partially removed by rinsing in diethyl pyrocarbonate (DEPC)-treated water. The gel was soaked in 0.05 N NaOH for 10 min to partially degrade RNA. This process facilitates transfer of RNAs to a nylon membrane. The gel was rinsed in DEPC-treated water and then soaked for 30-40 min in 20X SSC (Sambrook *et al.*, 1989). A nylon membrane (Hybond-N, Amersham) was prepared by soaking in DEPC-treated water and then immersing in 20 X SSC for 10 min. A piece of Whatman 3MM paper was placed on the top of a gel plate in a dish filled with 20 X SSC almost reaching the top of the support. The paper was allowed to become wet, and air bubbles between the paper and the support were eliminated. The gel was placed on the paper in an inverted position. Edges of the gel were surrounded with several pieces of parafilm to prevent direct flow of liquid from the dish to paper towels. A wet nylon membrane was placed on top of the gel. Two pieces of wet 3MM paper were put on the nylon membrane. Paper towels were stacked on the 3MM papers. A plastic plate was placed on the stack and weighed down with a heavy book (0.5-1 kg). RNAs were allowed to transfer to the membrane for 18 hours. Intermittently wet towels

were replaced. After removing the towels and papers, corresponding positions of the well in the gel were marked on the blotted membrane with a pencil. The nylon membrane was soaked in 6 X SSC for 5 min to eliminate any pieces of agarose and dried for 30 min at room temperature. The dried membrane was placed between two pieces of paper and baked at 80°C for 2 hours to immobilize the RNAs on the membrane.

A human multiple tissue and a human brain tissue blots were purchased from Clontech. According to the manufacturer's protocol, the Northern blots contain 2 μg of poly(A)$^+$ RNA per lane. RNA was run on a denaturing gel, transferred to a nylon membrane, and fixed by UV irradiation.

3.3b Hybridization of the multiple tissue blots

The rat multiple tissue blot was prehybridized with a solution containing 5 X SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 0.005 M Na_2EDTA, pH 7.4 adjusted with 10 N NaOH), 10 X Denhardt's solution (1 g Ficoll 400, 1 g polyvinylpyrrolidone, 1 g bovine serum albumin), 2 % SDS, 50 % formamide, and sheared salmon sperm DNA (100 μg/ml). This solution was prewarmed at 50°C to dissolve the SDS. Prehybridization was performed in 10 ml of this solution at 42°C for 4 hours. The solution was replaced once with fresh one prewarmed to 42°C.

The probe was synthesized using d39 insert as substrate, which encodes from the II-III loop to the untranslated 3' end of the Rb21-channel. If there are splice variants or homologous transcripts in the blots, the probe may detect those as well as Rb21-channel-transcripts. The probe was prepared as follows. The insert of d39 clone was digested,

purified, and used as a substrate to synthesize ^{32}P-labeled probe using the RadPrime DNA labeling system (Gibco BRL). The cDNA substrate was denatured by heating at 99°C for 5 min. The labeling reaction was performed by annealing random primers to the denatured DNA and extending them with 5 units of Klenow fragment of DNA polymerase I in the presence of 1 μM α-^{32}P-dCTP (3000Ci/mmol), 5 μM dATP, 5 μM dTTP, and 5 μM dGTP in a final volume of 50 μl. The radioactivity and percent incorporation of the synthesized probe was determined as described previously (Section 3.2c). The specific activity was 1.73×10^6 Ci/mmol. The probe was denatured by heating at 99°C for 5 min and then added into the hybridization solution.

Human multiple tissue blots obtained from Clontech were prehybridized for 4 hours and hybridized for 15 hours in a vinyl bag according to the same procedure explained above. Specific activity of the synthesized probe was 1.68×10^6 Ci/mmol.

Hybridized membranes were washed from low to high stringency conditions by increasing temperature and decreasing salt concentration of washing solution. For the first wash, membranes were washed in a 2X SSC and 0.1 % SDS solution at room temperature for 15 min with shaking. After being replaced with fresh solution, the temperature was slowly increased to 45-50°C for 20 min. At 50°C, the washing solution was next replaced with a 0.5X SSC and 0.1 % SDS solution. The membrane was washed for 20 min. The washing solution was next replaced with a 0.1X SSC and 0.1 % SDS solution. The final wash for the rat blot was in fresh 0.1X SSC solution and the temperature was increased to 68°C. The rat blot was washed in a high stringenct condition (0.1X SSC and 0.1 % SDS solution at 68°C), while the human blots were washed in a less stringent condition (0.5 X

SSC and 0.1 % SDS solution at 60°C), because the rat-specific probes may not be identical to human version transcripts.

Washed membranes were blotted on papers and dried at 37°C for 30 min. These membranes were exposed to an X-ray film in an intensifying cassette (X-Omatic cassette, Kodac) which was kept at -80°C. After 1-2 days, the exposed film was developed to determine how long an exposure was required to detect moderate signals. Human blots were exposed for 10 days and the rat blot was exposured for 12 days. The exposed films were fixed and developed using a QX-70 film processor (Konica).

3.4 Expression of the Rb21-channel in *Xenopus* oocytes and HEK 293 cells

Expression of the Rb21-channel was attempted in *Xenopus* oocytes and HEK 293 cells. *Xenopus* oocytes have been used as an excellent expression system for the study of cloned ion channels (genes.Hames and Higgins, 1985; Dascal, 1987). Oocytes are relatively big so that they can be easily injected with exogenous mRNA, cRNA, or plasmids containing cloned. However, not all foreign genes are expressed in oocytes. Moreover, they also have disadvantages such as expression of diverse native ion currents and slow clamping speed due to their size. For expression of the Rb21-channel in *Xenopus* oocytes, complementary RNA (cRNA) of the Rb21-channel was synthesized using T7 RNA polymerase, and then injected into oocytes. The two microelectrode voltage clamp method was applied to measure currents from injected oocytes.

Alternatively, HEK 293 cells were used as another expression system from which endogenous ion channel currents were rarely detected. Compared with *Xenopus* oocytes,

HEK cells have an advantage that the cell membrane can be clamped quickly using the whole cell patch clamp configuration. However, expression methods and recording currents need more time and skill. For expression of Rb21-channel, the pTracer vector (Invitrogen) containing the full-length cDNA of Rb21-channel was transfected using Lipofectamine reagent (Gibco BRL). The mammalian expression vector contains a cytomegalovirus promoter and a reporter gene expressing green fluorescence protein (GFP). Expressed currents were measured by the ruptured patch clamp technique.

3.4a Synthesis of cRNA of the Rb21-channel

Synthesis of cRNA of Rb21-channel was performed according to the manufacturer's protocol (Ambion). Briefly, the pGEM-3Z vector containing full-length cDNA of Rb21-channel was linearized by *Xba* I (5214) or *Sac* I (polylinker). Complementary RNA (cRNA) of Rb21-channel was transcribed *in vitro* from 1 μg of linearized plasmid DNA in a volume of 20 μl containing the following: 10 units of T7 phage RNA polymerase, 2.5 mM of ATP, 2.5 mM of CTP, 2.5 mM of TTP, 1.5 mM GTP, 8 mM capped GTP analog (7-methyldiguanosine triphosphate, $m^7G(5')$-ppp(5')G), 40 mM Tris-HCl (pH 7.2), 6 mM $MgCl_2$, 10 mM dithiothreitol, and 4 mM spermidine (Ambion MEGAscript Kit, Ambion, Austin, TX). The addition of 7-methylated GTP analogs on the 5' end of synthesized RNA can protect cRNA from RNases. The transcription reaction was performed at 37°C for 1.5-2 hours, followed by digestion of the template DNA with 2 units of DNase I at 37°C for 15 min. After adjusting the final volume of the reaction to 50 μl with H_2O, 25 μl of 7.5 M $LiCl_2$ solution was mixed with the reaction to precipitate the synthesized RNA. This mixture was

incubated at -20°C for 1 hour. The precipitated RNA was collected by centrifugation at 10,000 xg for 20 min. The pellet was washed with 70 % ethanol two times and dissolved with 20 µl of RNase-free water. The quantity of synthesized cRNA was determined spectrophotometrically. The quality was examined on an EtBr-stained denaturing agarose gel. The synthesized cRNA appeared as a single band of expected size.

Other cRNAs synthesized by the same method were as follows: α1C from the cDNA encoding the rabbit α1C (Wei *et al.*, 1991); α1E from the cDNA encoding the human α1E (Schneider *et al.*, 1994); β2 from the cDNA encoding the rat β2 (Perez-Reyes *et al.*, 1992); β4 from the cDNA encoding the rat β4 (Castellano *et al*, 1993b); and α2δ from the cDNA encoding the rabbit skeletal α2δ, a gift from Dr. Tsutomu Tanabe (Yale University). The α1C cDNA was the mutant clone ΔN60, which was truncated to remove 60 amino acids from the N-terminal end of the rabbit α1C cDNA.

3.4b Expression of the Rb21-channel in *Xenopus* oocytes

Female *Xenopus laevis* was purchased from Nasco (Fort Atkinson, WI). Several ovary lobes were removed surgically from the frog anesthetized by 0.1 % of ethyl 3-aminobenzoate solution. Ovary lobes were transferred into Ca^{2+}-free OR solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM N-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), pH 7.6 adjusted with 1 M NaOH, autoclaved, then supplemented with 50 mg/ml of gentamicin sulfate). They were manually torn into small clusters of about 10 oocytes. Oocytes were defolliculated by shaking in Ca^{2+}-free solution containing 2 mg/ml collagenase (Type IA, Sigma). Defolliculated oocytes (stage V-VI) in good condition were manually

selected under microscope and then incubated in SOS solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6 adjusted with 1 M NaOH, 2.5 mM of pyruvate, and 50 mg/ml of gentamicin sulfate) at 18°C for several hours or overnight for recovery. Fifty nl of cRNA (50 ng for Rb21-channel; 5 ng for α1C; 0.05-0.1 ng for α1E; 2.5 ng for α2; 2.5 ng for β2) diluted in 0.1 M KCl was injected into each oocyte using a Drummond Nanoject pipette injector (Parkway, PA) attached to a Narishige micromanipulator (Tokyo, Japan) under a dissecting microscope. As a positive control, either human α1E-cRNA alone or rabbit α1C-cRNA or Ca^{2+} channel auxiliary subunits β2 plus α2 were injected into oocytes. As a negative control, 50 nl of 0.1 M KCl was injected into oocytes.

3.4c Current measurement of oocytes injected with Rb21-channel cRNA

The two-electrode voltage clamp method was applied to measure currents from oocytes injected with Rb21-channel cRNA. Glass electrodes were pulled from capillaries (#6010, A-M systems, Everett, WA), using a Model P-97 pipette puller (Sutter, Instrument Co., Novato, CA). The tip resistance of voltage and current electrodes was 1.5-2.4 MΩ when filled with 3 M KCl solution. Proper shape and resistance of electrodes were obtained from a program whose parameters consist of temperature, pull, velocity, and time. Temperature values were set based on ramp tests, and values of other parameters were set by trial and error. The melting temperature of glass electrodes obtained by a ramp test was 270°C. The parameters of the program used to pull electrodes were as follow:

	Temperature	Pull	Velocity	Time
Step 1	317°C	-	75	150
Step 2	315°C	-	50	150
Step 3	215°C	-	30	-

Oocytes were impaled in SOS solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM pyruvic acid and 50 mg/L gentamicin sulfate). Oocytes were voltage-clamped using a two-electrode voltage clamp amplifier (OC-725B, Warner Instrument Corp.). After adjusting clamping speed, currents were measured in SOS medium by a series of depolarization voltage steps from a holding potential of -100 mV. Existence of Na^+ channel currents was checked in SOS solution which contains 100 mM Na^+. To measure Ca^{2+} channel currents, the bathing solution (SOS) was exchanged with 40 mM Ba^{2+} solution (40 mM $Ba(OH)_2$, 50 mM NaOH, 1 mM KOH, 5 mM HEPES, adjusted to pH 7.4 with methanesulfonate).

Data were collected at 2000 Hz using the pClamp 6 system (Axon Instrument, Foster city, CA) via a Digidata 1200 A/D converter (Axon Instrument, Foster city, CA). Data were filtered at 400 Hz (#902 Frequency Devices, Haverhill, MA). Leak currents was subtracted using a P/4 protocol. Experiments were performed at room temperature (20-22°C).

3.4d Expression of the Rb21-channel in Human Embryonic Kidney (HEK293) cells

Although *Xenopus* oocytes have been used as a good expression system, expression of exogenous channels is sometime dependent on endogenous factors in oocytes or coexpression of other exogenous factors. For example, the rabbit skeletal muscle Ca^{2+} channel α1S was not expressed in *Xenopus* oocytes, but in mouse L-cells and dysgenic

myotubules (Tanabe, *et al.*, 1988; Perez-Reyes, *et al.*, 1989). Another example is that antisense oligonucleotide depletion of endogenous Ca^{2+} channel β3 subunit in *Xenopus* oocytes inhibited the expression of the human Ca^{2+} channel α1E (Tareilus, *et al.*, 1997), suggesting that endogenous β3 subunit might be a required factor to express the α1E in oocytes. These studies illustrate the importance in using alternate expression systems. A modified HEK cell line, stably transfected with the SV40 large T-antigen (called tsA201 cell) was a gift from Dr. Richard Horn (Jefferson Medical College) (Margolskee *et al.*, 1993). If there is a simian virus 40 origin of replication in a transfected vector, binding of T-antigen to simian virus 40 origin allows replication of a transfected vector to high copy number. TsA201 cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco BRL), 10 % bovine calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The pTracer vector containing the full-length construct of Rb21-channel was transfected into tsA201 cells using Lipofectamine reagent (Gibco BRL) according to the manufacturer'protocol (Gibco BRL). One day before transfection, cells were plated on coverslips at a density of $1x10^5$ per 35 mm dish. Two μg of the plasmid was mixed with 100 μl of Opti-MEM serum free medium (Gibco BRL) in a tube. Six to eight μl of Lipofectamine reagent was mixed with 100 μl of Opti-MEM serum free medium in a separate tube. The two mixtures were combined and incubated at room temperature for 30 min, followed by addition of 800 μl of Opti-MEM serum free medium. Cells were rinsed twice with 2 ml of Opti-MEM serum free medium and then the plasmid-Lipofectamine mixture was applied on the cells. The transfection was performed by incubation for 5 hours at 37°C in an incubator supplied by compressed air containing 5 % CO_2. After 5 hours, 1 ml

of DMEM growth medium was added to cells. These cells were incubated at least 18 hours before current recordings. Currents were usually measured during the first and second days after transfection. As a positive control for exogenous channel expression in tsA201 cells, rat skeletal Na^+ channel μ1 (Trimmer, *et al.*, 1989) was transfected using the same procedure explained above. As a negative control for transfection in tsA201 cells, blank pTracer vectors not containing Rb21-channel cDNA were transfected. Cells transfected by pTracer vectors were identified by green fluorescence under the microscope. Alternatively, cells transfected by pTracer vectors were also checked by β-galactosidase staining with X-gal according to a protocol provided from Gibco BRL. Briefly, cells were washed with 2 ml of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 9.6 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, pH 7.4 adjusted with 0.1 N NaOH) containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$. Washed cells were fixed in 1 ml of fixative solution (PBS containing 2 % formaldehyde and 0.05 % glutaraldehyde) for 5 min at room temperature, followed by washing twice with 2 ml of PBS. These fixed cells were treated with 1 mg/ml X-gal in staining solution (1 X PBS, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$) and incubated 2 hours at 37°C.

3.4e Current measurement of tsA201 cells transfected with the Rb21-channel

Whole cell patch clamp configuration was applied to measure ionic currents in transfected cells (Hamill *et al.*, 1981). A coverslip containing tsA201 cells was positioned in a recording chamber on the stage of an inverted phase-contrast microscope (Nykon, Tokyo, Japan). Pipettes were pulled from TW-150-6 capillary tubing (World Precision

Instrument, Inc., Sarasota, FL) using a Model P-97 Flaming-Brown pipette puller (Sutter Instrument Co., Novato, CA). Pipettes were fire-polished using a heater set up with a manipulator (Narishige, Japan). These pipettes had a resistance of 1 to 2.5 MΩ when filled with an internal solution (55 mM CsCl, 75 mM $CsSO_4$, 10 mM $MgCl_2$, 0.1 mM EGTA, 10 mM HEPES, pH 7.2 adjusted with CsOH). The pipette was fixed with its holder, which was connected to the head stage of the amplifier. The bath was grounded by a Ag/AgCl pellet. The voltage offset between the pipette and the ground was adjusted using the pipette offset function. Cells were touched with the pipette while bathed in the external Tyrode solution (140 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, 5 mM HEPES, pH 7.4). Contact with the cell was detected by the decrement of applied 20 mV rectangular pulses. A smooth negative pressure was applied to obtain a gigaseal between the pipette and the cell membrane. The membrane was ruptured by strong negative suction or a DC pulse of 1.3 V using the Zap circuit of the Axopatch 200A amplifier. The capacity transient was minimized by adjusting the whole-cell capacitance compensation. Whole cell currents were amplified using an Axopatch 200A amplifier (Axon Instrument, Foster City, CA). The currents were recorded as digital data using a Digidata 1200 A/D converter and pClamp 6 software (Axon Instrument, Foster City, CA). Leak currents were subtracted by a P/4 protocol. Na^+ channel currents were measured in Tyrode solution containing 140 mM Na^+. To measure Ca^{2+} channel currents, the external solution was exchanged with a 10 mM barium solution (10 mM $BaCl_2$, 140 mM tetraethylammonium chloride, 5 mM CsCl, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with TEA-OH). The external solutions were perfused into the bath (0.2 ml) at a rate of 2-3 ml/min.

CHAPTER IV

RESULTS

4.1 Overview of RT-PCR cloning

As discussed in Chapter II, RT-PCR cloning might be one of the feasible approaches to clone a novel Ca^{2+} channel. This approach was applied to clone a T-type Ca^{2+} channel under the assumption that a T-type channel may share homology with mammalian high or primordial VACCs. If PCR primers derived from conserved sequences of reported high VACCs are homologous to the corresponding portion of a T-type channel, the PCR-primers would amplify a T-type channel sequence as well as high VACC sequences. PCR substrates were reverse transcribed from RNAs isolated from diverse cells from which T-type or primitive Ca^{2+} channel currents have been characterized. PCR products were separated, purified, subcloned, and sequenced. cDNA sequence of each PCR product was translated from all six possible reading frames. Deduced amino acid sequences of PCR products were manually compared with an alignment of reported Ca^{2+} channel $\alpha 1$ subunits and assigned to types of reported Ca^{2+} channel $\alpha 1$ subunits, or unidentified sequences unrelated to Ca^{2+} channel sequences, on the basis of their relative sequence homology. The unidentified sequences were compared with known sequences deposited in the Genbank.

4.1a Calcium channels of *Xenopus laevis* oocytes

Xenopus oocytes were chosen for RT-PCR analysis because they express T-type currents (Lacerda *et al.*, 1994). PCR substrate was reverse-transcribed from poly(A)$^+$ RNA of defolliculated oocytes. Degenerate PCR primers (C8 and C9) were designed based on conserved amino acid sequences in the domain I S3 and pore of reported high VACC α1 subunits. If T-type calcium channels contain conserved amino acid sequences found in regions of the high VACC α1 subunits, PCR primers would amplify cDNA fragments of T-type Ca^{2+} channels as well as high VACC cDNAs.

The expected sizes of PCR products were about 530 base pairs for L-type Ca^{2+} channels and 470 base pairs for non-L-type Ca^{2+} channels. The PCR products separated in an agarose gel showed two bands of the expected sizes. The PCR products were cloned, sequenced, and translated into amino acid sequences. The deduced sequences of the PCR products were compared to reported Ca^{2+} channel sequences. Forty-seven of 72 cloned sequences (named as XenoA1 to XenoA72) showed similarity with published Ca^{2+} channel α1 sequences, while the other 25 were not related to reported Ca^{2+} channels. The 47 sequences could be classified into 5 types: α1C, α1D, α1A, α1B, and α1E sequences. The amino acid sequences of representative clones (XenoA2, XenoA3, XenoA12, XenoA24, and XenoA25) for 5 groups were compared and aligned with reported Ca^{2+} channel sequences according to their relative similarity.

The alignment in figure 2 showed that XenoA12 and XenoA25 matched well with L-type Ca^{2+} channel sequences. XenoA25 had the highest sequence homology with rabbit α1C (cardiac L-type). Their sequence identity was 84.5 % (table 3). XenoA12 shared 80 %

sequence identity with rat α1D (neuroendocrine L-type). Based on sequence identity percentages calculated in table 3, I concluded that XenoA25 is a cardiac L-type Ca^{2+} channel sequence, while XenoA12 is a neuroendocrine L-type Ca^{2+} channel sequence of *Xenopus laevis*.

According to relative sequence homology, XenoA2 was aligned with rabbit α1B (N-type) (figure 2). They shared 87.9 % sequence identity which was the highest value among homologous percentages, suggesting that XenoA2 is a frog isoform of N-type channel sequences. Interestingly, three other N-type sequences (XenoA14, XenoA28, and XenoA29) were cloned, whose sequences were almost identical to one another. All of the different amino acids between the four sequences were not due to the third nucleotide specific change of each codon. One of the possible explanation for the multiple forms of N-type sequences was that *Xenopus laevis* is a tetraploid animal (Kobel and Pasquier, 1986). This fact explains that there could be at least two sets of transcripts in an oocyte. Moreover, even if RNA was isolated from defolliculated oocytes, contamination by follicular cells sticking on oocytes can not be ruled out. A second possible explanation was that multiple forms of N-type sequences could result from PCR errors. Possible error rates by *Taq* DNA polymerase were reported to be less than 1/400 (Innis *et al.*, 1990). However, mismatch rates between the four N-type sequences ranged from 6/420 to 15/420. These significant differences suggested that the PCR cloning of multiple N-type sequences could not be explained only by errors of Taq polymerase. Another possibility was that RNA editing processes might partially contribute to the existence of the multiple N-type sequences (Bass, 1997).

XenoA24 was aligned with rabbit α1E based on relative sequence similarity. Their

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                 ┌─────────IS3─────────┐                                  ┌─────────IS4─────────┐
α1Srb   :GWNVLDFIIVFLGVFTAILEQVNVIQSNTAPMSSKGAGLDVKALRAFRVLRPLRLVSGVP
α1Crb   :GWNLLDFIIVVVGLFSAILEQATK.ADGANALGGKGAGFDVKALRAFRVLRPLRLVSGVP
XenoA25:GWNVLDFIIVVVGLFSAILEQATK.ADGGSAIEGKGAGFDVKALRAFRVLRPLRLVSGVP
α1Drt   :GWNLLDFVIVIVGLFSVILEQLTKETEGGNHSSGKSGGFDVKALRAFRVLRPLRLVSGVP
XenoA12:GWNVIDFVIVVIGLFSVILEQFSHKPGDAHHMSGKPGGFDVKALRAFRVLRPLRLVSGVP
All-Ls  :GWN-LDF-IV--G-F--ILEQ------------K--G-DVKALRAFRVLRPLRLVSGVP
α1Arb   :GWNVMDFVVVLTGILATVGTE.................FDLRTLRAVRVLRPLKLVSGIP
α1Brb   :GWNVMDFVVVLTGILATAGTD.................FDLRTLRAVRVLRPLKLVSGIP
XenoA14:GWNVLDFVVVLTGILATAGTD.................FDLRTLRAVRVLRPLKLVSGIP
XenoA28:  NVIDFVVVLTGILATAGTD.................FDLRTLRAVRVLRPLKLVSGIP
XenoA2  :   LDFVVVLTGILATAGTD.................FDLRTLRAVRVLRPLKLVSGIP
XenoA29:GWNVMDFVVLLTGILATAGTD.................FDLRTLRAVRVLRPLKLVSGIP
α1Erb   :GWNVMDFIVVLSGILATAGTHFNTH.............VDLRTLRAVRVLRPLKLVSGIP
Xenoa24:AWNVLDFIVVLSGILATAGTHFNTH.............VDLRTLRAVRVLRPLKLVSGIP
Non-Ls  :GWNVMDF-VVL-GILAT-GT------------------DLRTLRAVRVLRPLKLVSGIP
XenoA3  :GWNVMDFLVVLSGIVASTGPE.................FDLRTLRAVRVLRPLKLVSGIP


                                  ┌─────────IS5─────────┐
α1Srb   :SLQVVLNSIFKAMLPLFHIALLVLFMVIIYAIIGLELFKGKMHKTCYYIGTDIVATVEN
α1Crb   :SLQVVLNSIIKAMVPLLHIALLVLFVIIIYAIIGLELFMGKMHKTCYNQEGVADVPAED
XenoA25:SLQVVLNSIIKAMVPLLHIALLVLFVIIIYAIIGLELFMGKMHKTCYFL.TNGY.PEEE
α1Drt   :SLQVVLNSIIKAMVPLLHIALLVLFVIIIYAIIGLELFIGKMHKTCFFADS..DIVAEE
XenoA12:SLHIVLNSIMKAMVPLLHIALLVLFVIIIYAIIGLELFIGRMHKTCFYIG..SDLVSEE
All-Ls  :SLQVVLNSI-K-M-PL-HIALLVLF--IIYAIIGLELF-GKMHKTC-----------E-
α1Arb   :SLQVVLKSIMKAMIPLLQIGLLLFFAILIFAIIGLEFYMGKFHTTCFEEGTDDIQGES
α1Brb   :SLQVVLKSIMKAMVPLLQIGLLLFFAILMFAIIGLEFYMGKFHKACFPNSTDPDPVG
XenoA14:SLQVVLKSIMKAMVPLLQIGLLLFFAIVMFAIIGLEFYMGKFHKACFSDDSDE.PVG
XenoA28:SLQVVLKSIMKAMVPFLQIGLSLFFVIVMFAIIGLEFYMGKFHKACFSDDSDE.PVG
XenoA2  :SLQVVLKSIMKAMVPLLQIGLLLFFAIVMFAIIGLELYMGKFHKACFSDDSDE.PVG
XenoA29:SLQVVLKSIMKAMVPLLQIGLLLFFAIVMFAIIGLEFYMGKFHKACFSDDSDE.PVG
α1Erb   :SLQIVLKSIMKAMVPLLQIGLLLFFAILMFAIIGLEFYSGKLHRACFVNNSGVLEGFDP
Xenoa24:SLQIVLKSIMKAMVPLLQIGLSLFFAILMFAIIGLEFYSGKLHKACYRNNSGMGLEPDP
Non-Ls  :SLQ-VLKSIMKAM-PLLQIGLLLFFAIL-FAIIGLEFY-GK-H--CF------------
XenoA3  :SLQVVLKSIMKAMIPLLQIGLLLFFAILIFAIIGLDFYIGKFHTSCY.DISGEDLKV


                                                          ┌─────────PORE─────────┐
α1Srb   :EKPSPCAR.TGSGRPCTINGSECRGGWPGPNHGITHFDNFGFSMLTVYQCITMEGWT
α1Crb   : DPSPCALETGHGRQC.QNGTVCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWT
XenoA25:  PSPCALESNNGRHC.ANGTVCRSGWAGPKHGITNFDNFAFAMLTVFQCITMEGWT
α1Drt   : DPAPCAF.SGNGRQCAANGTECRSGWVGPNGGITNFDNFAFAMLTVFQCITMEGWT
XenoA12: EPAPCAF.SGHGRECLLNNTECRGKWEGPNGGITNFDNFFAMLTVFQCITMEGWT
All-Ls  :--P-PCA---G-GR-C--NG--C--GW-GP--GIT-FDNF-F-MLTV-QCITMEGWT
α1Arb   :  PAPCG.TEEPARTCP.NGTRCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGWT
α1Brb   :  DFPCG.KEAPARLCE.GDTECREYWAGPNFGITNFDNILFAILTVFQCITMEGWT
XenoA14:  DFPCG.EELPARLCE.NGTTCRVYWKGPNFGITNFDNILFAVLTVFQCITMEGWT
XenoA28:  DFPCG.EELPARLCE.NGTTCRVYWKGPNFGITNFDNILFAVLTVFQCITMEGWT
XenoA29:  DFPCG.EELPARLCE.NGTSCRVYWKGPNFGITNFDNILFAVLTVFQCITMEGWT
XenoA2  :  DFPCG.EELPARLCE.NGTSCRVYWKGPNFGITNFDNLLFAVLTVFQCITMEGWT
α1Erb   :  PHPCG....VQG.CP.AGYECKD.WIGPNDGITQFDNILFAVLTVFQCITMEGWT
Xenoa24:  PHPCG....VPR.CP.AGYECRE.WIGPNDGITQFDNILFAVLTVFQCITM____
Non-Ls  :  --PCG--------C------C---W-GPN-GIT-FDNILFA-LTVFQCITMEGWT
XenoA3  :  EVLCG.NDSSSRHCP.NNTYCLSKWEGPNNGITQFDNILFAILTVFQCITMEGWT
```

Figure 2. Alignment of *Xenopus* oocyte calcium channel α1 subunits to reported calcium channel α1 subunits. Deduced amino acid sequences of calcium channel α1 subunits cloned from *Xenopus* oocytes are aligned with the corresponding region of the mammalian channels on the basis of sequence homology. Sources of reported channels are as follows: α1Srb is from a rabbit skeletal L-type (M23919; Tanabe *et al.*, 1987); α1Crb is from a rabbit cardiac L-type (X15539; Mikami *et al.*, 1989); α1Drt is from a rat neuroendocrine L-type (M57682; Hui *et al.*, 1991); α1Arb is from a rabbit P/Q-type (X57476; Mori *et al.*, 1991); α1Brb is from a rabbit N-type (Fujita *et al*, 1993); and α1Erb is from a rabbit R-type channel (X67855; Niidome *et al*, 1992). Amino acids are given in the one letter code. Conserved amino acids of L-type and non-L-type channels are included below aligned sequences and marked as bold letters, and non-conserved amino acids are marked as dashes. Distinguishing amino acids found in oocyte calcium channel sequences are marked as bold letters. Underlined amino acids in the initial and terminal regions of sequences are deduced amino acids of forward and reverse primers (C8 and C9) used in PCR.

	α1S-rabbit	α1C-rabbit	α1D-rat	α1A-rabbit	α1B-rabbit	α1E-rabbit
α1S-rabbit	100	71	71	53	53	50
α1C-rabbit		100	78	58	57	55
α1D-rat			100	58	57	57
α1A-rabbit				100	81	72
α1B-rabbit					100	75
α1E-rabbit						100
XenoA25	66.3	**84.5**	77.5	59.5	56.7	53.9
XenoA12	66.9	70.6	**80.0**	57.8	55.6	56.2
XenoA14	51.1	59.1	61.1	80.3	**89.4**	71.5
XenoA2 97.9% to 14	51.8	59.9	61.8	78.9	**87.9**	70.1
XenoA28 97.7% to 14 95.5% to 2	47.8	56.2	58.1	77.0	**86.6**	67.9
XenoA29 98.6% to 14 97.9% to 2 96.2% to 28	51.1	59.1	61.1	79.6	**88.7**	70.8
XenoA24	48.0	50.3	59.2	69.7	73.5	**90.0**
XenoA3	51.0	54.9	54.7	**76.2**	71.2	65.8

Table 3. Percent sequence identity between *Xenopus* oocyte Ca^{2+} channels and reported Ca^{2+} channels. Percentages of sequence identity were calculated using the DNASIS program. Portions of PCR primers were deleted in the calculation of homology percentage. The highest homology values are marked as bold letters. Sources for reported calcium channel sequences are given in the legend of figure 2.

sequence identity was 90% which was the highest value among similarity percentages, suggesting that XenoA24 is a *Xenopus* isoform of R-type channel sequences.

XenoA3 shared the highest homology of 76.2 % with rabbit α1A (P/Q-type), suggesting that XenoA3 is a P/Q-type Ca^{2+} channel sequence. However, XenoA3 might be a new type because the sequence homology percentage was lowest. Moreover, XenoA3 contained distinctive amino acids which were not found in rabbit α1A or any other α1 subunits in the regions of IS3 and the loop between IS5 and I pore. Another possible interpretation was that the α1A sequence of *Xenopus laevis* might have diverged more during evolution, resulting in less conserved sequence compared with other Ca^{2+} channel types.

The first PCR cloning experiment detected all the Ca^{2+} channel α1 sequences except skeletal L-type Ca^{2+} channel α1 sequence. One of the possible reasons why the PCR primers (C8 and C9) did not amplify a *Xenopus laevis* version of skeletal L-type Ca^{2+} channel α1 sequence was that the frog L-type channel might contain poorly conserved amino acid sequences found in domain IS3 and domain I pore of mammalian VACC α1 subunits. Thus, another PCR cloning was carried out to test whether *Xenopus* oocytes express skeletal L-type Ca^{2+} channel transcripts using the L-type specific PCR primers (C5 and C6) designed on the basis of conserved amino acid sequences in putative membrane spanning segment 6 (S6) in repeats III and IV of published L-type Ca^{2+} channel α1 subunits.

PCR products amplified by L-type specific primers appeared as a band of about 950 base pairs on an agarose gel. The size of PCR products was consistent with the expected size, suggesting that the PCR primers specifically amplified L-type Ca^{2+} channel cDNAs from oocyte cDNAs. PCR products were cloned, sequenced, and translated. Three types of

L-type Ca^{2+} channel sequences were observed. These amino acid sequences were aligned with reported Ca^{2+} channel sequences according to their relative sequence similarity (figure 3). Sequence identity percentages between oocyte L-type sequences and published L-type channel sequences were calculated in table 4. Based on these homology percentages, oocyte L-type sequences were classified as follows: XenoL8 was a skeletal L-type (α1S) ; XenoL36 was a cardiac L-type (α1C); XenoL17 was a neuroendocrine L-type (α1D) sequence of *Xenopus laevis*. Cloning of three classes of L-type channel sequences from *Xenopus* oocytes indicates that oocytes express mRNAs for three types of L-type Ca^{2+} channels.

Taken together with the previous PCR cloning using C8 and C9 primers, these studies showed that *Xenopus* oocytes express at least 6 types of high VACCs. However, a novel Ca^{2+} channel sequence, such as a candidate for a T-type Ca^{2+} channel sequence, was not cloned from *Xenopus* oocyte RNA.

4.1b Calcium channels of bovine adrenal glomerulosa cells

Adrenal glomerulosa cells are reported to express T-type Ca^{2+} channels which are involved in secreting aldosterone, a salt-regulating hormone (McCarthy *et al.*, 1993). RT-PCR cloning was applied to clone a T-type Ca^{2+} channel from bovine adrenal glomerulosa RNA using the C8 and C9 primers. PCR products were cloned into pGEM-T vectors (Promega) which were transformed into *E. coli* JM 109 competent cells. Plasmids were isolated from seventy-two transformants. Whether plasmids contained PCR products was checked by restriction enzyme digestion. PCR products were sequenced, translated and compared with reported Ca^{2+} channel sequences.

```
          IIIS6──────┐
α1SRB   :FFMMNIFVGFVIVTFQEQGETEYKNCELDKNQRQCVQYALKARPLRCYIPKNPYQYQVWYVVTS
Xenoα1S:FFMMNIFVGFVIVTFQEQGEQEYKNCELDKNQRQCVQYALKAHPLRRYIPKNQYQYKIWYLVTS
α1CRB   :FFMMNIFVGFVIVTFQEQGEQEYKNCELDKNQRQCVEYALKARPLRRYIPKNQHQYKVWYVVNS
XENOα1C:FFMMNIFVGFVIVTFQEQGEQEYKNCELDKNQRQCVEYALKARPLRRYIPKNQYQYKVWYVVSS
α1DRT   :FFMMNIFVGFVIVTFQEQGEKEYKNCELDKNQRQCVEYALKARPLRRYIPKNPYQYKFWYVVNS
XENOα1D:FFMMNIFVGFVIITFRAQGEQEYKNCELDKNQRQCVEYALKAQPLRRYIPKAKCQYKVWYMVNS

            ┌──────IVS1──────┐                ┌──────IVS2──────┐
α1SRB   :SYFEYLMFALIMLNTICLGMQHYHQSEEMNHISDILNVAFTIIFTLEMILKLLAFKARGYFGDP
Xenoα1S:SYFEYLMFALIMLNTISLGMKHYGQTAEFSHLSDILNVAFTGMFTVEMILKLAAFKAKGYFGDP
α1CRB   :TYFEYLMFVLILLNTICLAMQHYGQSCLFKIAMNILNMLFTGLFTVEMILKLIAFKPKGYFSDP
XENOα1C:TYFEYLMFVLILLNTICLAMQHYGQSCSFKEAMNILNMLFTGLFTVGMILKLIAFKPKGYFSDP
α1DRT   :SDFEYMMFVLIMLNTLCLAMQHYEQSKMFNDAMDILNMVFTGVFTVEMVLKVIAFKPKGYFSDA
XENOα1D:TGFEYIMFVLILLNTVALAMQHYEQSKPFNYAMDILNMVFTGLFTVEMVLKLMAFRPKHYFCDA

          ┌──────IVS3──────┐                                ┌──────IVS4─
α1SRB   :WNVFDFLIVIGSIIDVILSEIDTFLASSGGLYCLGGGCGNVDPDESARISSAFFRLFRVMRLI
Xenoα1S:WNVFDFLIVIGSIIDVILSEIDTPDPVVTPGT----------EDTSRISITFFRLFRVLRLV
α1CRB   :WNVFDFLIVIGSIIDVILSETNPAEHTQCSPSMNA--------EENSRISITFFRLFRVMRLV
XENOα1C:WNVFDFLIVIGSVVDVILSETNPGEHAQCSSSMNV--------EENSRISITFFRLFRVMRLV
α1DRT   :WNTFDSLIVIGSIIDVALSEADPSDSENIPLPTATPGNS----EESNRISITFFRLFRVMRLV
XENOα1D:WNTFDALIVVGSLVDIAVTEVNNGWHVGESS------------EDSSRISITFFRLFRVMRLV

          ┌──────┐                      ┌──────IVS5──────┐                ┌─
α1SRB   :KLLSRAEGVRTLLWTFIKSFQALPYVALLIVMLFFIYAVIGMQMFGKIALVDGTQINRNNNFQ
Xenoα1S:KLLSRGEGVRTLLWTFIKSFQALPYVALLIVMLFFIYAVIGMQVFGKIAMVDGTQINRNNNFQ
α1CRB   :KLLSRGEGIRTLLWTFIKSFQALPYVALLIVMLFFIYAVIGMQVFGKIALNDTTEINRNNNFQ
XENOα1C:KLLSRGEGIRTLLWTFIKSFQALPYVALLIVMLFFIYAVIGMQVFGKIALNDTTSLNRNNNFQ
α1DRT   :KLLSRGEGIRTLLWTFIKSFQALPYVALLIAMLFFIYAVIGMQMFGKVAMRDNNQINRNNNFQ
XENOα1D:KLLSKGEGIRTLLWTFIKSFQALRYVALLIAMIFFIYAVIGMQTFGKIAMQDGSQINRNNNFQ

          IV Pore loop──────┐                                ┌──────IVS6──
α1SRB   :TFPQAVLLLFRCATGEAWQEILLACSYGKLCDPESD--YAPGEEYTCGTNFAYYYFISFYMLW
Xenoα1S:TFPQAVLVLFRCATGEAWQEILLACSYGKLCDPKSD--FLPGEEYTCGTSFAYFYFISFYMLW
α1CRB   :TFPQAVLLLFRCATGEAWQDIMLACMPGKKCAPESEPHNSTEGETPCGSSFAVFYFISFYMLW
XENOα1C:TFPQAVLLLFRCATGEAWQEVMLACLPNKPCAPESE----TSEEK-CGSSFAVFYFISFYMLW
α1DRT   :TFPQAVLLLFRCATGEAWQEIMLACLPGKLCDPDSD--YNPGEEYTCGSNFAIVYFISFYMLW
XENOα1D:TFPQAVLLLFRCATGEAWQDIMLASLPGKRCDSESD--YGPGEEFTCGSNFAIVYFISFYMLW
```

Figure 3. Alignment of *Xenopus* oocyte L-type calcium channel α1 subunits to reported L-type calcium channel α1 subunits. Deduced amino acid sequences of the three L-type calcium channel α1 subunits cloned from *Xenopus* oocytes are aligned with the corresponding channel type on the basis of sequence homology (refer to table 2). Amino acids are given in the one letter code. Underlined amino acids in the initial and terminal regions of the alignment are the deduced amino acids of forward and reverse primers (C5 and C6).

	α1S-RT	α1C-RB	α1D-RT	XenoL8	XenoL36	XenoL17
XenoL8	**81**	76.5	76.7		76.4	72.1
XenoL36	71.7	**90.5**	76.4	76.4		74.4
XenoL17	67	74.1	**78.4**	72.1	74.4	

Table 4. Percentages of sequence identity between *Xenopus* oocyte L-type Ca^{2+} channel sequences and reported Ca^{2+} channel. Identical percentages were calculated using the DNASIS program (Hitachi). Sources of reported calcium channels were given in the legend of figure 2. Portions of PCR primers were included in the calculation of homology percentage. Highest homology values are marked as bold letters.

Twenty-three of 72 sequences (named as Glos1 to Glos72) showed similarity with known Ca^{2+} channel $\alpha 1$ sequences. On the contrary, the DNA and deduced amino acid sequences of the other 49 were not homologous to reported Ca^{2+} channels. These translated sequences did not contain consensus sequences found in S4 and pore of reported VACCs. The 23 sequences similar to Ca^{2+} channel $\alpha 1$ sequences were classified with Glos20, Glos48, Glos6, and Glos8 sequences as representative members. In figure 4, the representative sequences were aligned with reported Ca^{2+} channel sequences based on their relative sequence similarity. Glos20 was almost identical to the rabbit cardiac L-type Ca^{2+} channel sequence, suggesting that Glos20 is a bovine version of cardiac L-type Ca^{2+} channel sequences. Glos48 was identical to Glos20 except missing amino acids from the end portion of IS3 to before IS4 (32 amino acids). Glos48 also missed the 4 amino acids found in the loop between IS5 and I pore of Glos20. This four amino acid deletion in Glos48 might be caused by an alternative splicing process because this portion is one of possible splicing sites identified from the genomic structure of human $\alpha 1C$ (Soldatov, 1994). However, the IS3 deletion in Glos48 might result from a PCR error or an RNA splicing error, but probably not from an RNA alternative splicing. In any case, I predict that the translated protein without this big portion would not fold properly, and would not form a functional channel.

Glos6 shared the highest homology (96.9 %) with the rabbit neuroendocrine L-type Ca^{2+} channel sequence ($\alpha 1D$), suggesting that Glos6 is a bovine $\alpha 1D$ sequence. Based on the sequence homology, Glos8 was classified as a bovine version of P/Q-type Ca^{2+} channel sequences ($\alpha 1A$), while Glos13 was classified as a bovine isoform of R-type Ca^{2+} channel sequences ($\alpha 1E$).

```
                 ┌────────IS3────────┐                                ┌────────IS4────────┐
α1SRB   :GWNVLDFIIVFLGVFTAILEQVNVIQSNTAPMSSKGAGLDVKALRAFRVLRPLRLVSGVP
α1CRB   :GWNLLDFIIVVVGLFSAILEQATK.ADGANALGGKGAGFDVKALRAFRVLRPLRLVSGVP
Glos20  :GWNLLDFIIVVVGLFSAILEQATK.AEGANALGGKGAGFDVKALRAFRVLRPLRLVSGVP
Glos48  :GWNVID.............................FDVKALRAFRVLRPLRLVSGVP
α1DRB   :GWNLLDFVIVIVGLFSVILEQLTKETEGGNHSSGKSGGFDVKALRAFRVLRPLRLVSGVP
Glos6   :GWNVIDFVIVIVGLFSVILEQLTKETEGGNHSSGKSGGFDVKALRAFRVLRPLRLVSGVP
All-Ls  :GWN-LDF-IV--G-F--ILEQ------------K--G-DVKALRAFRVLRPLRLVSGVP
α1ARB   :GWNVMDFVVVLTGILATVGTE.............FDLRTLRAVRVLRPLKLVSGIP
Glos8   :GWNVIDFVVVLTGILATVGTE.............FDLRTLRAVRVLRLLKLVSGIP
α1BRB   :GWNVMDFVVVLTGILATAGTD.............FDLRTLRAVRVLRPLKLVSGIP
α1ERB   :GWNVMDFIVVLSGILATAGTHFNTH..........VDLRTLRAVRVLRPLKLVSGIP
Glos13  :GWNVLDFIVVLSGILATAGTHFNTH..........VDLRTLRAVRVLRPLKLVSGIP
Non-Ls  :GWNVMDF-VVL-GILAT-GT----------------DLRTLRAVRVLRPLKLVSGIP

                                ┌────────IS5────────┐
α1SRB   :SLQVVLNSIFKAMLPLFHIALLVLFMVIIYAIIGLELFKGKMHKTCYYIGTDIVATVENEK
α1CRB   :SLQVVLNSIIKAMVPLLHIALLVLFVIIIYAIIGLELFMGKMHKTCYNQEGVADVPAEDD
Glos20  :SLQVVLNSIIKAMVPLLHIALLVLFVIIIYAIIGLELFMGKMHKTCYNQEGIADVPAEDD
Glos48  :SLQVVLNSIIKAMVPLLHIALLVLFVIIIYAIIGLELFMGKMHKTCYNQEGIA....EDD
α1DRB   :SLQVVLNSIIKAMVPLLHIALLVLFVIIIYAIIGLELFIGKMHKTCFFADS..DIVAEED
Glos6   :SLQVVLNSIIKAMVPLLHIALLVLFVIIIYAIIGLELFIGKMHKTCFFADS..DMVAEED
All-Ls  :SLQVVLNSI-K-M-PL-HIALLVLF--IIYAIIGLELF-GKMHKTC----------E-D-
α1ARB   :SLQVVLKSIMKAMIPLLQIGLLLFFAILIFAIIGLEFYMGKFHTTCFEEGTDDIQGES
Glos8   :SLQVVLKSIMKAMIPLLQIGLLLFFAILIFAIIGLEFHMGKFHTTCFEEGTDDIQGES
α1BRB   :SLQVVLKSIMKAMVPLLQIGLLLFFAILMFAIIGLEFYMGKFHKACFPNSTDPDPVG
α1ERB   :SLQIVLKSIMKAMVPLLQIGLLLFFAILMFAIIGLEFYSGKLHRACFVNNSGVLEGFDP
Glos13  :SLQIVLKSIMKAMVPLLQIGLLLFFAILMFAVIGLEFYSGKLHRACFMNNSGILEGFDP
Non-Ls  :SLQ-VLKSIMKAM-PLLQIGLLLFFAIL-FA-IGLEFY-GK-H--CF----------

                                                ┌────────PORE────────┐
α1SRB   :PSPCAR.TGSGRPCTINGSECRGGWPGPNHGITHFDNFGFSMLTVYQCITMEGWT
α1CRB   :PSPCALETGHGRQC.QNGTVCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWT
Glos20  :PSPCALETAHGRQC.QNGTVCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWT
Glos48  :PSPCALETAHGRQC.QNATVCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWT
α1DRB   :PAPCAF.SGNGRQCAANGTECRSGWVGPNGGITNFDNFAFAMLTVFQCITMEGWT
Glos6   :PAPCAF.SGNGRQCTANGTESRSGWAGPNGGITNFDNFAFAMLTVFQCITMEGWT
All-Ls  :P-PCA-----GR-C--N------GW-GP--GIT-FDNF-F-MLTV-QCITMEGWT
α1ARB   :PAPCG.TEEPARTCP.NGTRCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGWT
Glos8   :PAPCG.TEEPARTCP.NGTKCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGWT
α1BRB   :DFPCG.KEAPARLCE.GDTECREYWAGPNFGITNFDNILFAILTVFQCITMEGWT
α1ERB   :PHPCG....VQG.CP.AGYECKD.WIGPNDGITQFDNILFAVLTVFQCITMEGWT
Glos13  :PHPCG....VQG.CP.AGYECRD.WIGPNDGITQFDNILFAVLTVFQCITMEGWT
Non-Ls  :--PCG--------C------C---W-GPN-GIT-FDNILFA-LTVFQCITMEGWT
```

Figure 4. Alignment of bovine adrenal glomerulosa Ca^{2+} channel $\alpha 1$ subunits to reported Ca^{2+} channel $\alpha 1$ subunits. Translated amino acid sequences of RT-PCR cloned calcium channel cDNAs from bovine adrenal glomerulosa RNA are aligned with published calcium channel sequences on the basis of sequence similarity. Sources for reported calcium channel sequences are given in figure 2. Conserved amino acids of L-type and non-L-type channels are included below aligned sequences and marked as bold letters, and non-conserved amino acids are marked as dashes. Unique amino acids found in bovine glomerulosa calcium channel sequences are marked as bold letters. The single letter codes for amino acids are used. Underlined amino acids in initial and terminal regions of sequences are deduced amino acids of forward and reverse primers (C8 and C9) used in PCR.

Taken together, PCR cloning detected α1C, α1D, α1A, and α1E cDNA sequences from bovine adrenal glomerulosa RNA. These results suggested that bovine adrenal glomerulosa cells might express the following high VACCs; cardiac L-, neuroendocrine L-, P/Q-, and R-type Ca^{2+} channels. However, N-type (α1B) and a candidate of T-type Ca^{2+} channel sequences were not detected.

4.1c A putative calcium channel sequence of *Paramecium tetraurelia*

The same experimental methods were applied to clone a Ca^{2+} channel sequence from a protozoan, *Paramecium tetraurelia*. Total RNA isolated from the primitive animal was reverse-transcribed into cDNA which was used as PCR substrate. The C8 and C9 PCR primers were used to amplify Ca^{2+} channel sequences by PCR. Gel electrophoresis of the reaction showed a product of 410 base pairs, which was smaller than the expected sizes (530 base pairs for L-type Ca^{2+} channels; 470 base pairs for non-L-type Ca^{2+} channels). PCR products were cloned, sequenced, and compared with reported Ca^{2+} channel sequences.

The hypothesis was that mammalian VACCs share conserved amino acid sequences with novel Ca^{2+} channels including T-type and primitive Ca^{2+} channels. If a *Paramecium* Ca^{2+} channel contains similar sequences to C8 and C9 primers derived from domain I S3 and pore of reported Ca^{2+} channels, primers would amplify a Ca^{2+} channel sequence from the protozoan animal. A significant sequence similarity existed between the counterparts (S3 and pore) of domain I and III. Therefore, the C8 and C9 primers may possibly amplify S3 to pore regions of domain III as well as those of domain I. In this experiment, Para 5, a putative *Paramecium* Ca^{2+} channel sequence, shared higher homology with sequences of

domain III than those of domain I so that Para 5 was aligned, then compared with corresponding sequences of the domain III of reported Ca^{2+} channels (figure 5). However, Para 5 may correspond to domain I. To prove whether the clone sequence is a part of domain I or III, the full-length cDNA sequence of the *Paramecium* channel should be cloned, translated, and compared with those of mammalian Ca^{2+} channels. If the *Paramecium* sequence pertains to domain III, that could be a clue supporting an evolutionary theory of four-domain Ca^{2+} channels, which were evoled from one-domain primitive channels such as voltage-activated K^+ channels via double duplication (Hille, 1992).

Another interesting point was that in ciliate protozoa such as *Paramecium*, glutamine can be also encoded by TAA and TGA, which encode a stop codon in other living organisms (Caron and Meyer, 1985). The TAA found in the end of Domain IIIS5 was marked as q which presumably encodes a glutamine residue.

Sequence identity percentages between Para 5 and reported Ca^{2+} channel sequences ranged between 29 to 41 % (table 5). Even if Para 5 showed relatively low sequence homology with reported Ca^{2+} channel sequences, the sequence contained amino acid residues conserved in the domain III S4 and pore regions of mammalian Ca^{2+} channels. These data suggested that Para 5 is a cDNA fragment of a *Paramecium* Ca^{2+} channel.

4.1d Calcium channels of rat heart detected by RT-PCR cloning

T-type Ca^{2+} channel currents as well as L-type were reported to exist in cardiac myocytes (Bean, 1985; Nilius *et al.*, 1985; Hagiwara *et al.*, 1988). To clone the cardiac T-type Ca^{2+} channel, RT-PCR cloning was applied using C8 and C9 PCR primers. PCR

```
          ┌--------IIIS3------┐                    ┌--------IIIS4------┐
α1SRB   :YFNILDLLVVAVSLISMGLE---SST----ISVVKILRVLRVLRPLRAINRAKGLKHVV
α1CRB   :YFNILDLLVVSVSLISFGIQ---SSA----INVVKILRVLRVLRPLRAINRAKGLKHVV
α1DRB   :YFNLLDMLVVGVSLVSFGIQ---SSA----ISVVKILRVLRVLRPLRAINRAKGLKHVV
Para5   :GWNVLDFIIVLVSFVDIYVE---GVD----LSFVKILRLLRTLRDLRFISHNKSMKLLV
α1ARB   :LWNILDFIVVSGALVAFAFT---GNSKGKDINTIKSLRVLRVLRPLKTIKRLPKLKAVF
α1BHS   :LWNILDFIVVSGALVAFAFS---G-SKGKDINTIKSLRVLRVLRPLKTIKRLPKLKAVF
α1ERB   :LWNILDFVVVVGALVAFALANALGTNKGRDIKTIKSLRVLRVLRPLKTIKRLPKLKAVF
           * **   *                    * ** ** ****  *      *

                        ┌--------IIIS4------┐
α1SRB   :QCVFVAIRTIGNIVLVTTLLQFMFACIGVQLFKGKFFSCNDLSKMTEEECRGYYYVYKD
α1CRB   :QCVFVAIRTIGNIVIVTTLLQFMFACIGVQLFKGKLYTCSDSSKQTEAECKGNYITYKD
α1DRB   :QCVFVAIRTIGNIMIVTTLLQFMFACIGVQLFKGKFYRCTDEAKSNPEECRGLFILYKD
Para5   :NTLINSISGIVNVGVVVILVWMMFAILGVNVqKGKMWYCDTGSDNWFHVQQCAQEN---
α1ARB   :DCVVNSLKNVFNILIVYMLFMFIFAVVAVQLFKGKFFHCTDESKEFEKDCRGKYLLY--
α1BHS   :DCVVNSLKNVLNILIVYMLFMFIFAVIAVQLFKGKFFYCTDESKELERDCRGQYLDY--
α1ERB   :DCVVTSLKNVFNILIVYKLFMFIFAVIAVQLFKGKFFYCTDSSKDTEKECIGNYVDH--
                    *   *  *    **   *   ***   *

                              ┌-------IIIPORE------┐
α1SRB   :GDPTQMELRPRQWIHNDFHFDNVLSAMMSLFTVSTFEGWPQLLYRAIDSNEEDM  1032
α1CRB   :GEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSIDSHTEDK  1163
α1DRT   :GDVDSPVVRERIWQNSDFNFDNVLSAMMALFTVSTFEGWPALLYKAIDSNGENI  1119
Para5   :----------GVWRNRDMNFDNVFSGFLTLFIISTMEGW
α1ARB   :-EKNEVKARDREWKKYEFHYDNVLWALLTLFTVSTGEGWPQVLKHSVDATFENQ  1487
α1BHS   :-EKEEVEAQPRQWKKYDFHYDNVLWALLTLFTVSTGEGWPMVLKHSVDATYEEQ  1383
α1ERB   :-EKNKMEVKGREWKRHEFHYDNIIWALLTLFTVSTGEGWPQVLQHSVDVTEEDR  1380
                  *       **       **  ** ***
```

Figure 5. Alignment of a putative *Paramecium tetraurelia* calcium channel α1 subunit to reported calcium channel α1 subunits. Para5 is a putative *Paramecium tetraurelia* calcium channel α1 cDNA fragment amplified by RT-PCR. The deduced sequence of Para5 is aligned with the most homologous portions (Domain III S3 to Domain III pore) of published calcium channel α1 sequences. Sources of the published sequences are as follows: α1SRB is from a rabbit skeletal L-type (M23919; Tanabe *et al.*, 1987); α1CRB is from a rabbit cardiac L-type (X15539; Mikami *et al.*, 1989); α1DRT is from a rabbit neuroendocrine L-type (M57682; Hui *et al.*, 1991); α1ARB is from a rabbit P/Q-type (X57476; Mori *et al.*, 1991); α1BHS is from a human N-type (M94172; Williams *et al.*, 1992a); α1ERB is from a rabbit R-type calcium channel α1 subunit (X67855; Niidome *et al.*, 1992). Amino acids are given in the one letter code. Bolded are amino acids found in a putative *Paramecium* calcium channel and conserved in either L- or non-L-type calcium channels. Common residues between a putative *Paramecium tetraurelia* calcium channel α1 and all reported calcium channel α1 sequences are marked as asterisks (*) below the aligned sequences. Underlined amino acids in initial and terminal regions of sequences are deduced amino acids of forward and reverse primers (C8 and C9) used in PCR.

	α1S-RB	α1C-RB	α1D-RB	α1A-RB	α1B-HS	α1E-RB
Para5	41	34	37	29	35	33

Table 5. Sequence identity percentages between Para5 and reported calcium channels. The deduced amino acid sequence of Para5 was compared to amino acid sequences of the α1S, α1C, α1D, α1A, and α1E of rabbit, and the α1B of human using the DNASIS program (Hitachi). Portions representing PCR primers are included in the calculation of homology percentage. Sources for mammalian calcium channels are given in the legend of figure 5.

products were cloned, sequenced, and compared with published Ca^{2+} channel sequences. Either 5'-end or 3'-end of cloned PCR products were sequenced to classify cloned channel sequences into their types.

According to their sequence homology, translated sequences were classified and positioned below corresponding rabbit or rat Ca^{2+} channel sequences (figure 6). The clone, Ratht8, contained the same amino acid sequence as the rabbit cardiac Ca^{2+} channel $\alpha 1$ subunit (α1Crt). This result indicates that Ratht8 is a rat cardiac L-type Ca^{2+} channel fragment. Interestingly, Ratht24 was identical to the sequence of the rat neuroendocrine L-type Ca^{2+} channel $\alpha 1$ subunit (α1Drt), suggesting that rat heart expressed α1D transcripts. This fact was consistent with the previous report that α1D mRNA was detected heart by Northern blot analysis (Yaney *et al.*, 1992). Furthermore, recent studies suggested that α1D channels contribute to the cardiac L-type current, since an antibody developed against the external pore portion in domain IV of mammalian α1D decreased cardiac Ca^{2+} channel currents 30 %(Wyatt *et al.*, 1997). Ratht17 was almost identical α1Arb (rabbit P/Q-type Ca^{2+} channel $\alpha 1$ subunit), indicating that Ratht17 is a rat P/Q-type $\alpha 1$ sequence. Ratht38 was classified into a R-type Ca^{2+} channel sequence, since Ratht38 showed identical amino acid sequence to rabbit α1E.

Taken together, four types of Ca^{2+} channel sequences were detected from rat heart by PCR cloning; cardiac L-, neuroendocrine L-, P/Q-, and R-type Ca^{2+} channel sequences. But a novel channel sequence was not cloned. Although cardiac L-type and T-type Ca^{2+} channel currents were identified biophysically (Bean, 1985; Nilius *et al.*, 1985; Hagiwara *et al.*, 1988), α1D, α1A, and α1E sequences cloned from rat heart raised the following questions:

```
                 ┌─────IS3─────┐                              ┌─────IS4─────
α1Srb    : GWNVLDFIIVFLGVFTAILEQVNVIQSNTAPMSSKGAGLDVKALRAFRVLRPLRLV≈
α1Crb    : GWNLLDFIIVVVGLFSAILEQATK.ADGANALGGKGAGFDVKALRAFRVLRPLRLV≈
Ratht8   : GWNLLDFIIVVVGLFSAILEQATK.ADGANALGGKGAGFDVKALRAFRVLRPLRLV≈
α1Drt    : GWNLLDFVIVIVGLFSVILEQLTKETEGGNHSSGKSGGFDVKALRAFRVLRPLRLV≈
All-Ls   : GWN-LDF-IV--G-F--ILEQ----------K--G-DVKALRAFRVLRPLRLV≈
α1Arb    : GWNVMDFVVVLTGILATVGTE.................FDLRTLRAVRVLRPLKLV≈
α1Brb    : GWNVMDFVVVLTGILATAGTD.................FDLRTLRAVRVLRPLKLV≈
α1Erb    : GWNVMDFIVVLSGILATAGTHFNTH.............VDLRTLRAVRVLRPLKLV≈
Ratht38  :  WNVLDFIVVLSGILATAGTHFNTH.............VDLRTLRAVRVLRPLKLV≈
Non-Ls   : GWNVMDF-VVL-GILAT-GT------------------DLRTLRAVRVLRPLKLV≈


                                                  ┌─────PORE─────┐
α1Srb    : ≈PSPCAR.TGSGRPCTINGSECRGGWPGPNHGITHFDNFGFSMLTVYQCITMEGWT
α1Crb    : ≈PSPCALETGHGRQC.QNGTVCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWT
Ratht8   : ≈PSPCALETGHGRQC.QNGTVCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWT
α1Drt    : ≈PAPCAF.SGNGRQCAANGTECRSGWVGPNGGITNFDNFAFAMLTVFQCITMEGWT
Ratht24  : ≈PAPCAF.SGNGRQCAANGTECRSGWVGPNGGITNFDNFAFAMLTVFQCITMEGWT
consenL  : ≈P-PCA---G-GR-C--NG--C--GW-GP--GIT-FDNF-F-MLTV-QCITMEGWT
α1Arb    : ≈PAPCG.TEEPARTCP.NGTRCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGWT
Ratht17  : ≈PAPCG.TEEPARTCP.NGTKCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGW
α1Brb    : ≈DFPCG.KEAPARLCE.GDTECREYWAGPNFGITNFDNILFAILTVFQCITMEGWT
α1Erb    : ≈PHPCG....VQG.CP.AGYECKD.WIGPNDGITQFDNILFAVLTVFQCITMEGWT
Non-Ls   : ≈--PCG--------C------C---W-GPN-GIT-FDNILFA-LTVFQCITMEGWT
```

Figure 6. Alignment of rat heart calcium α1 subunits to reported calcium channel α1 subunits. Since either 5'- or 3'-end of PCR products were sequenced, only partial amino acid sequences of PCR products cloned from rat heart RNA are aligned with corresponding calcium channel types on the basis of sequence homology. Sources for published calcium channel sequences are given in the legend of figure 2. The ommitted portion is marked as "≈" in the alignment. Consensus amino acids of L-type and non-L-type channels are marked as bold letters below aligned sequences, while non-conserved amino acids are marked as dashes. Amino acids are given in the one letter code. Underlined amino acids in initial and terminal regions of the sequences are deduced amino acids of forward and reverse primers (C8 and C9) used in PCR.

where are the three types of channels expressed in heart, and what are their physiological functions?

4.1e Calcium channels of rat brain detected by RT-PCR cloning

Multiple types of high VACCs including L-, N-, P/Q-, and R-type have been identified from tissues in brain based on electrophysiological and pharmacological criteria (Randall and Tsien, 1995; Dolphin, 1995). Expression studies of cloned Ca^{2+} channel $\alpha 1$ subunits and their auxiliary subunits have mimicked most of high VACC currents described from neuronal cells (Perez-Reyes and Schneider, 1994). However, T-type Ca^{2+} channels have not been cloned yet. RT-PCR cloning methods similar to previous experiments were applied to clone a T-type channel from brain. The C8-C9 PCR primers were used to amplify Ca^{2+} channel sequences from brain cDNA.

Because cloned PCR products were same or almost identical to corresponding type of reported rabbit Ca^{2+} channel sequences, either 5'- or 3'- end of each clone was partially sequenced to identify what types of channel sequences were cloned. The deduced sequences were aligned with reported Ca^{2+} channel sequences in figure 7. On the basis of sequence homology, cloned sequences were classified as follows: Ratbr70 is a cardiac L-type channel sequence ($\alpha 1C$); Ratbr45 is a neuroendocrine L-type channel sequence ($\alpha 1D$); Ratbr35 and 36 are P/Q-type channel sequences ($\alpha 1A$); Ratbr9 and 26 are N-type channel sequences ($\alpha 1B$); Ratbr25 and 28 are R-type channel sequence ($\alpha 1E$).

Taken together, five classes of Ca^{2+} channel sequences ($\alpha 1C$, $\alpha 1D$, $\alpha 1A$, $\alpha 1B$, and $\alpha 1E$) were cloned from brain by PCR. These results were consistent to previous reports that

```
                 ┌─────────IS3──────────┐                                  ┌─────────IS4──────────
α1Srb   : GWNVLDFIIVFLGVFTAILEQVNVIQSNTAPMSSKGAGLDVKALRAFRVLRPLRLV≈
α1Crb   : GWNLLDFIIVVVGLFSAILEQATK.ADGANALGGKGAGFDVKALRAFRVLRPLRLV≈
Ratbr70: GWNLLDFIIVVVGLFSAILEQATK.ADGANALGGKGAGFDVKALRAFRVLRPLRLV≈
α1Drt   : GWNLLDFVIVIVGLFSVILEQLTKETEGGNHSSGKSGGFDVKALRAFRVLRPLRLV≈
All-Ls  : GWN-LDF-IV--G-F--ILEQ------------K--G-DVKALRAFRVLRPLRLV≈
α1Arb   : GWNVMDFVVVLTGILATVGTE.................FDLRTLRAVRVLRPLKLV≈
Ratbr35: GWNVIDFVVVLTGILATVGTE.................FDLRTLRAVRVLRPLKLV≈
α1Brb   : GWNVMDFVVVLTGILATAGTD.................FDLRTLRAVRVLRPLKLV≈
Rarbr9  : GWNVIDFVVVLTGILATAGTD.................FDLRTLRAVRVLRPLKLV≈
α1Erb   : GWNVMDFIVVLSGILATAGTHFNTH.............VDLRTLRAVRVLRPLKLV≈
Ratbr25: GWNVLDFIVVLSGILATAGTHFNTH.............VDLRTLRAVRVLRPLKLV≈
Non-Ls  : GWNVMDF-VVL-GILAT-GT------------------DLRTLRAVRVLRPLKLV≈


                                                      ┌──────────PORE──────────┐
α1Srb   : ≈PSPCAR.TGSGRPCTINGSECRGGWPGPNHGITHFDNFGFSMLTVYQCITMEGWT
α1Crb   : ≈PSPCALETGHGRQC.QNGTVCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWT
α1Drt   : ≈PAPCAF.SGNGRQCAANGTECRSGWVGPNGGITNFDNFAFAMLTVFQCITMEGWT
Ratbr45: ≈PAPCAF.SGNGRQCAANGTECRSGWVGPNGGITNFDNFAFAMLTVFQCITMEGWT
consenL: ≈P-PCA---G-GR-C--NG--C--GW-GP--GIT-FDNF-F-MLTV-QCITMEGWT
α1Arb   : ≈PAPCG.TEEPARTCP.NGTRCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGWT
Ratbr36: ≈PAPCG.TEEPARTCP.NGTKCQPYWEGPNNGITQFDNILFAVLTVFQCITMEG
α1Brb   : ≈DFPCG.KEAPARLCE.GDTECREYWAGPNFGITNFDNILFAILTVFQCITMEGWT
ratbr26: ≈DFPCG.KEAPARLCD.SDTECREYWPGPNFGITNFDNILFAILTVFQCITMEGWT
α1Erb   : ≈PHPCG....VQG.CP.AGYECKD.WIGPNDGITQFDNILFAVLTVFQCITMEGWT
Ratbr28: ≈PHPCG....VQG.CP.AGYECKD.WIGPNDGITQFDNILFAVLTVFQCITMEGWT
Non-Ls  : ≈--PCG--------C------C---W-GPN-GIT-FDNILFA-LTVFQCITMEGWT
```

Figure 7. Alignment of PCR cloned rat brain calcium α1 subunits to reported calcium channel α1 subunits. Since 5' or 3' end of PCR-products were sequenced, only partial amino acid sequences cloned from rat brain RNA are aligned with corresponding published channel types on the basis of sequence homology. Sources for published calcium channel sequences are given in the legend of figure 2. The omitted portion is marked as "≈" in the alignment. Conserved amino acids of L-type and non-L-type channels are marked as bold letters below aligned sequences, while non-conserved amino acids are marked as dashes. Amino acids are given in the one letter code. Underlined amino acids in initial and terminal regions of the sequences are deduced amino acids of forward and reverse primers (C8 and C9) used in PCR.

five types of high VACC mRNAs except skeletal L-type channel were detected in brain by Nothern blot analysis and *in situ* hybridization (Perez-Reyes and Schneider, 1994). However, a novel channel sequence was not cloned from rat brain by PCR cloning.

4.1f Calcium channels of NIE-115 cells detected by RT-PCR cloning

C8-C9 primers have detected Ca^{2+} channels from *Xenopus* oocytes, *Paramecium tetraurelia*, bovine adrenal glomerulosa, and rat heart and brain, but primers did not detect any novel sequence, such as a candidate of T-type Ca^{2+} channels. One possible reason that C8-C9 primers did not amplify a novel sequence was that T-type channels contain poorly conserved amino acid sequences in the domain IS3 and I pore of reported Ca^{2+} channels. Another possibility was that PCR using the C8-C9 primers might have rarely amplified low VACC sequences compared with high VACC sequences, because most of the tissues used for previous experiments express T-type Ca^{2+} channels less abundantly than high VACCs.

To compensate for these possible weak points, first, I sequenced many clones containing PCR products to increase the possibility of cloning a novel Ca^{2+} channel sequence. Second, four new pairs of PCR primers were newly designed on the basis of conserved sequences in the three domains of reported Ca^{2+} channels (table 2). Third, T-type currents share similar properties with voltage-activated Na^+ channel currents in terms of low-voltage activation and relatively fast inactivating kinetics by highly depolarizing test pulses. Assuming that T-type channels might contain consensus sequences found in voltage-activated Ca^{2+} and Na^+ channels, the T1 and T2 primer pair (table 2) was designed from sequences conserved in both Ca^{2+} and Na^+ channels. Fourth, cDNA reverse-transcribed from

NIE-115 neuroblastoma RNA was used as PCR substrate because undifferentiated cells were reported to express T-type Ca^{2+} channel currents exclusively (Liévano *et al.*, 1994).

PCR products amplified by T1-T2 primers were cloned, sequenced, and translated into amino acid sequences. Translated sequences were compared with reported channel sequences, and aligned with Ca^{2+} and Na^{+} channel sequences according to their relative homology (figure 8). The deduced sequence of NIE-6 was identical to a cardiac L-type sequence of murine (α1Cmus). This result indicates that NIE-6 is a α1C sequence. NIE-16 was classified into a N-type sequence because the sequence showed highest homology of 97 % with the mouse N-type sequence. In addition to two types of Ca^{2+} channel sequences, a Na^{+} channel sequence (NIE-2) similar to the rat brain sodium channel 1 was amplified by the T1-T2 primers.

Three other pairs of PCR primers, T3-T4, T3-T5, and T6-T7, were used to amplify Ca^{2+} channel sequences from NIE-115 neuroblastoma cDNA. PCR products amplified by these primers were summarized in table 6. The T3-T4 primers detected two types of Ca^{2+} channel sequences, which were classified as cardiac L- and neuronal N-type sequences based on their similarity. T3-T5 primers amplified a N-type Ca^{2+} channel sequence. T6-T7 primers also detected a N-type Ca^{2+} channel sequence.

Taken together, the new sets of PCR primers detected two types of Ca^{2+} channel sequences and a Na^{+} channel as well. Despite extensive studies, however, the designed primers did not amplify a candidate of novel Ca^{2+} channel sequences. These results suggested that T-type Ca^{2+} channel sequences might have poor homology to reported high VACC sequences.

```
        --IIS5--┐                    ┌-------IIpore----
α1Srb   :GMQLFGGRYDFEDTEVRRSNFDNFPQALISVFQVLTGE
α1Cmus  :GMQLFGGKFNFDEMQTRRSTFDNFPQSLLTVFQVLTGE
N1E-6   :GMQLFGGKFNFDEMQTRRSTFDNFPQSLLTVFQILTGE
α1Drt   :GMQLFGGKFNFDETQTKRSTFDNFPQALLTVFQILTGE
All-Ls  :GMQLFGG---F------RS-FDNFPQ-LL-VFQILTGE
α1Art   :GMQLFGGQFNFDE.GTPPTNFDTFPAAIMTVFQILTGE
α1Bmus  :GMQLFGGQFNFQD.ETPTTIFDTFPAAILTVFQILTGE
N1E-16  :GMQLFGGQFNFQD.ETPTTNFDTFPAAILTVFQILTGE
α1Ert   :GMQLFGGRFNFND.GTPSANFDTFPAAIMTVFQILTGE
Non-Ls  :GMQLFGG-FNF----TP---FDTFPAAI-TVFQILTGE

Na1-rt  :GMQLFGKSYKDCVCKIATDCKLPRWHMNDFFHSFLIVFQILTGE
N1E2    :GMQLFGKSYKECVCKIASDCSLPRWHMHDFFHSFLIVFQILTGE
```

Figure 8. Alignment of NIE-115 neuroblastoma Ca^{2+} and Na^{+} channel α1 subunits to reported channel α1 subunits. Translated sequences of PCR products from NIE-115 RNA are aligned with reported calcium and sodium channel sequences. Sources for published channel sequences are as follows: α1Srb is a rabbit skeletal L-type (Genbank access number; M23919); α1Cmus is a murine cardiac L-type (L06233); α1Drt is a rat neuroendocrine L-type (M57682); α1Art is a rat neuronal P/Q-type (M64373); α1B is a rat neuronal N-type (M92905); α1Erat is a rat R-type calcium channel (L15453); and Na1-rt is a sodium channel type 1 of rat brain (X03638). Conserved amino acids of L-type and non-L-type channels are marked as bold letters below aligned sequences, while non-conserved amino acids are marked as dashes. Distinct amino acids found in calcium and sodium channel sequences of NIE-115 neuroblastoma are marked as bold letters. Amino acids are given in the one letter code. Underlined amino acids in initial and terminal regions of sequences are deduced amino acids of forward and reverse PCR primers (T1 and T2).

PCR primer pairs	Total clones sequenced	N-type Ca^{2+} channel	Cardiac L-type channel	Na^{+} channel	Unidentified sequences
T1-T2	46	16	9	8	12
T3-T4	51	19	1	0	31
T3-T5	24	4	0	0	20
T6-T7	48	23	0	0	25

Table 6. Summary of RT-PCR cloning from NIE-115 neuroblastoma RNA. Four pairs of PCR primers were used to amplify calcium channel sequences. Translated sequences of PCR products were compared with reported channel sequences. Based on sequence homology, deduced sequence of each PCR product was classified into either a calcium channel, a sodium channel, or an unidentified sequence which is not related to voltage-activated ion channels.

4.2 A putative calcium channel sequence cloned from rat brain

PCR primers derived from conserved amino acid sequences of reported Ca^{2+} channels amplified published high VACC sequences, and even a Na^{+} channel sequence as well as a putative *Paramecium* Ca^{2+} channel sequence. However, PCR primers failed to amplify a novel Ca^{2+} channel sequence. One of the possible reasons was that low VACCs might have considerably diverged from high VACCs so that they contain poorly conserved amino acid sequences in those portions which were used to design the PCR primers.

Low and high VACCs might have diverged from an ancestral type of Ca^{2+} channel during evolution (Hille, 1992). In the previous experiments, all the PCR primers were designed from conserved sequences in reported high VACCs, under the hypothesis that low VACCs may contain consensus sequences found in high VACCs. Another possible hypothesis based on the Ca^{2+} channel evolution theory is that primitive Ca^{2+} channels found in simple species may contain consensus sequences found in low and high VACCs.

Information about primitive Ca^{2+} channel sequences was obtained in two ways. First, RT-PCR was attempted to clone primitive Ca^{2+} channel sequences from simple organisms such as *Paramecium.* In a previous experiment, the C8-C9 primers amplified a putative Ca^{2+} channel sequence from *Paramecium tetraurelia.* Even if S4 and pore regions of the primitive sequence were homologous to mammalian Ca^{2+} channels, the portions did not contain enough information to design new PCR primers. The reasons were that corresponding codons to amino acids in S4 were very degenerate, and pore sequence was partially from C9 primer. Second, primitive calcium channel sequences were searched from the Genbank where numerous sequences from expressed mRNAs and genome DNA sequencing projects

of diverse species were deposited. For example, 2.2 Mbases of 100 Mbase genome of *Caenorhabditis elegans* were sequenced and deposited in the Genbank (Wilson *et al.*, 1994). Using the BLAST (Basic Local Alignment Search Tool; Altschul *et al.*, 1990) program, calcium channel-like sequences were searched for in the Genbank. A candidate for a putative Ca^{2+} channel sequence (Genbank access number; U40419) was found in a C27f2 cosmid, where a chromosomal DNA segment of *Caenorhabditis elegans* was cloned. The reason that C27f2 sequence could be a candidate for a novel Ca^{2+} channel was that the structure of the putative Ca^{2+} channel consisted of four domains, each of which contained 6 putative membrane spanning segments and a pore loop. However, the novel sequence showed low sequence homology to a L- and a non-L- types of Ca^{2+} channel sequences found in *C. elegans* (Genbank access numbers; D67507, D68412). Furthermore, the putative Ca^{2+} channel sequence in C27f2 could not be classified as some type of calcium and sodium channels due to its relative low sequence homology. This led to the hypothesis that the *C. elegans* sequence is a candidate for a low voltage-activated calcium channel.

To clone a mammalian isoform of the putative *C. elegans* channel, three pairs of PCR primers were designed from the amino acid sequence of the putative *C. elegans* channel (table 2). If designed primers have sequence homology to a counterpart channel of rat, designed primers would amplify a rat version of the *C. elegans* channel sequence. Among three pairs of degenerate PCR primers, T15 and T16 primers derived from amino acid sequences in the domain IV S5 and pore of the C27f2 sequence amplified a Ca^{2+} channel-like sequence from rat brain RNA. The translated sequence (named as Rb21) was aligned with corresponding portions of mammalian Ca^{2+} channel sequences in figure 9. The sequence

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                -IVS5-┐                  ┌---------IVpore-----
α1SRb       : GMQMFGKIALVDGTQINRNNNFQTFPQAVLLLFRCATGEAWQ
α1CRb       : GMQVFGKIALNDTTEINRNNNFQTFPQAVLLLFRCATGEAWQ
α1DHm       : GMQMFGKVALRDNNQINRNNNFQTFPQAVLLLFRCATGEAWQ
α1ARb       : GMQVFGNIGIEDEFQITEHNNFRTFFQALMLLFRSATGEAWH
α1BHm       : GMQVFGNIALDDDTSINRHNNFRTFLQALMLLFRSATGEAWH
α1ERb       : GMQVFGNIRLDEESHINRHNNFRSFFGSLMLLFRSATGEAWQ
C.el C27f   : GVVLFGMVKY--GQAVGKHVNFRNGREALVVLFRSVTGEDWN
Rb21        : GVVLFGMVKY--GENINRHANFSSAGKAITVLFRIVTGEDWN
                *    **          **** **      *    ***  *** *
```

Figure 9. Alignment of the Rb21-PCR product to published Ca^{2+} channel and *C. elegans* C27f2 sequences. A pair of PCR primers were designed from underlined portions of *C.el* C27f which is a putative calcium channel sequence in a *Caenorhabditis elegans* cosmid, C27f2 (Genbank access number; CEL27f2). The primers amplified Rb21 cDNA from rat brain RNA. Sources for aligned channel sequences are as follows: α1SRb is a skeletal L-type channel of rabbit (M23919; Tanabe *et al.*, 1987); α1CRb is a cardiac L-type channel of rabbit (L04569; Schultz *et al.*, 1993); α1DHm is a neuroendocrine L-type channel of human (M76558; Williams *et al.*, 1992b); α1ARb is a P/Q-type channel of rabbit (X57476; Mori *et al*, 1989); α1BHm is a N-type channel of human (M94172; Williams *et al.*, 1992a); α1ERb is a R-type calcium channel of rabbit (X67855; Niidome *et al*). Identical amino acid residues between *C.el* C27f and Rb21 were highlighted. Residues of Rb21 showing identity to more than three groups out of six calcium channels are marked with asterisks (*) under the alignment.

identity between the Rb21 and C27f2 sequences was 65 %, suggesting that Rb21 was a rat isoform of the C27f2 sequence. Rb21 shared about 40 % sequence homology with cloned mammalian Ca^{2+} channel sequences. Rb21 contained amino acid residues conserved in domain IV pores of published Ca^{2+} channels. These results suggested that Rb21 was a rat version of the putative *C. elegans* ion channel sequence and a candidate of novel ion channel sequences.

4.3 Cloning of the Rb21 full-length cDNA from a rat brain cDNA library

The process of library screening and schematic diagram of the full-length cDNA of Rb21 are shown in figure 10. To clone the full-length cDNA of Rb21, a rat brain cDNA library (Clontech) was screened repeatedly using ends of positive clones as probes.

The full-length cDNA was constructed from the following overlapping clones: rbIII7, f2, g2, and d39. Postive clones were ligated into the pGEM-3Z vector (Promega) using the following restriction enzyme sites: *Eco* RI (-64), *Xma* I (1505), *Eco* RI (2037), *Nco* I (3394), and *Sac* I (polylinker).

The cDNA sequence of the full-length clone could be obtained by connecting the overlapping sequences of rbIII7, f2, g2, and d39 clones. Nevertheless, the initial full-length construct of the Rb21-channel was sequenced more than 90 % in both directions to check whether the construct contained point mutations. Deletion or additon of nucleotides were not detected from the whole construct. The full length cDNA was subcloned into pTracer (Invitrogen) for expressing the putative channel in mammalian cells.

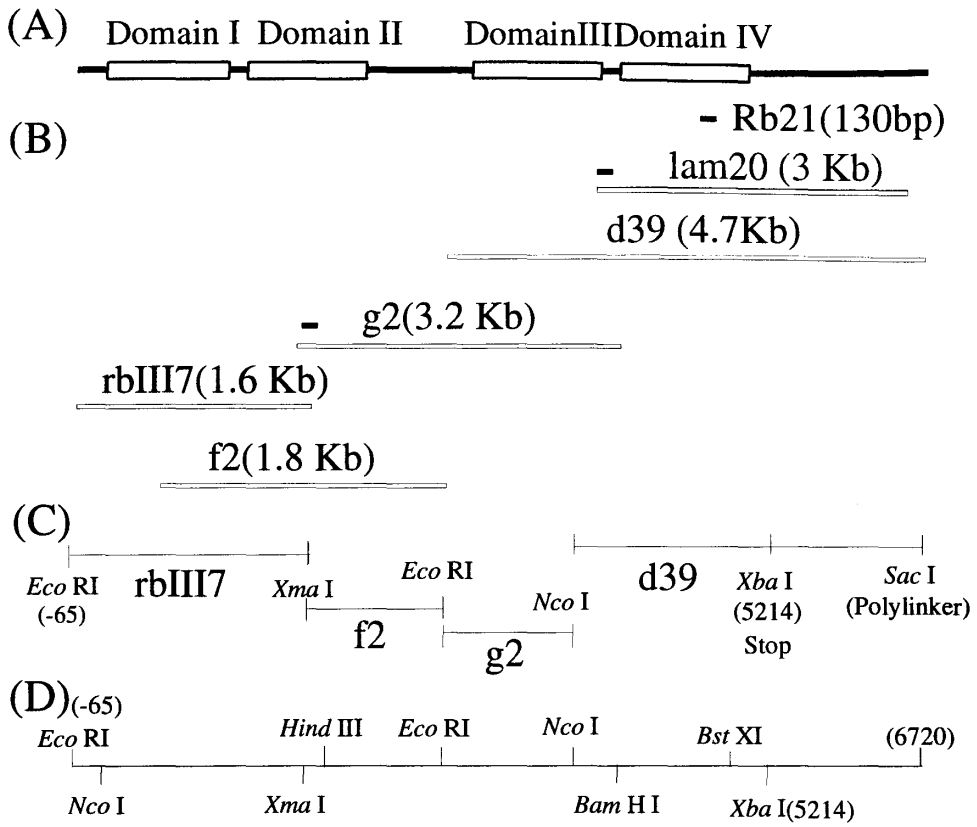


Figure 10. Library screening and full-length cloning strategies of the Rb21-channel. The four domain structure of Rb21-channel is schematically drawn in (A). Processes of library screenings and detected positive clones are shown in (B). Small black bars represent cDNAs used as probes for library screenings. To construct the full-length cDNA of the Rb21 channel, four cDNA fragments from four positive clones are joined and cloned into the pGEM-3Z (Promega) and the pTracer (Invitrogen) (C). The open reading frame stops at 5214 (*Xba* I), followed by untranslated 3'-end. Restriction enzyme sites used for making probes and constructing the full-length cDNA are mapped in (D).

4.4 Structure of the Rb21-channel

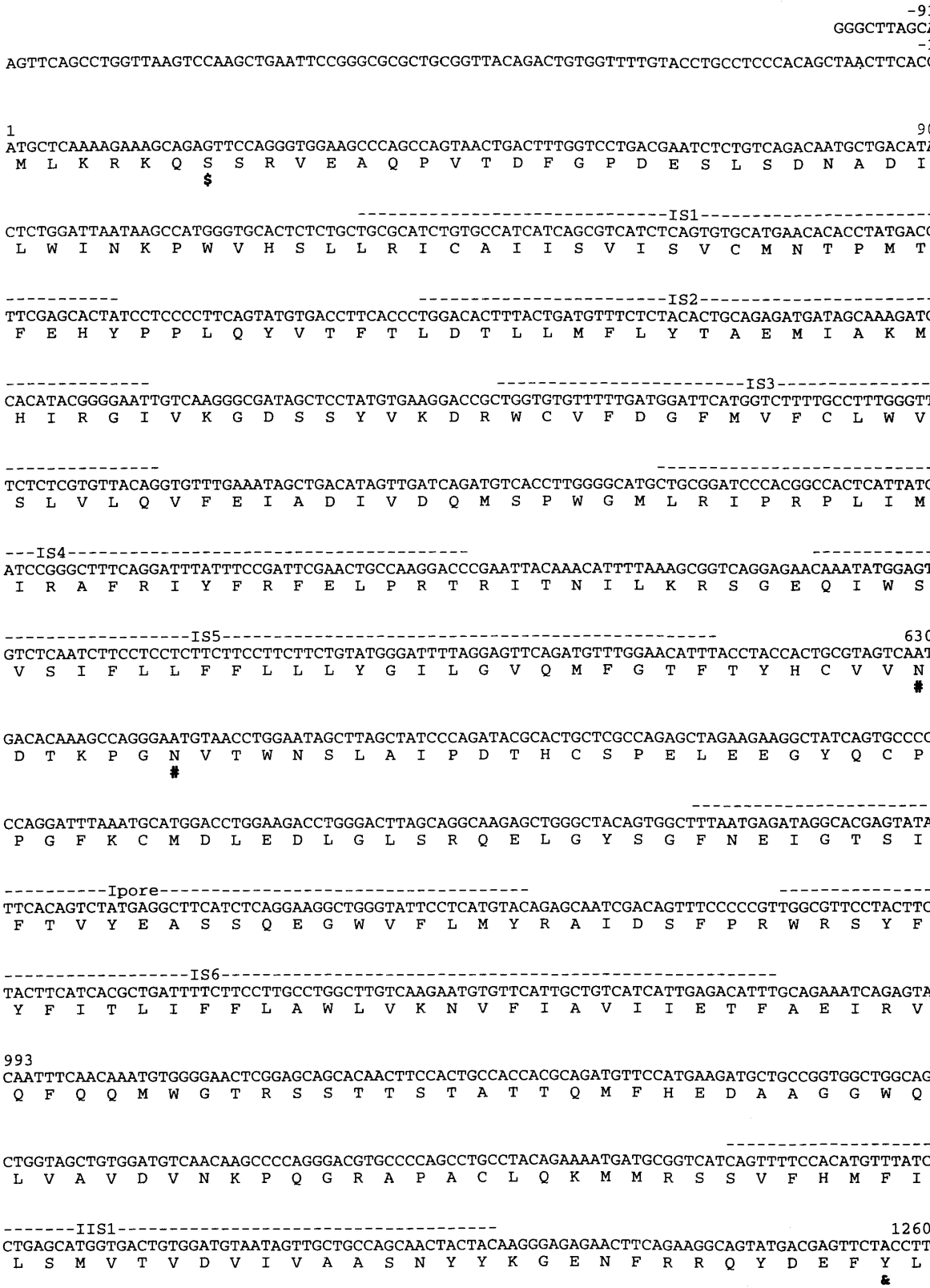
4.4a Complementary DNA and translation of the Rb21-channel

The full length cDNA of the Rb21-channel was translated into amino acids in figure 11. The cloned cDNA of Rb21-channel contains 6820 bases. An open reading frame begins with methionine (ATG) and ends with a stop codon (TAG). The first ATG in the full-length cDNA was preceded by a stop codon at -66 in the 5'-untranslated region. The flanking DNA sequence (ACACCATGC) of the underlined initiation codon is very similar to reported Kozak sequence of CC(A/G)CCATGG, which is a consensus sequence for translation start identified from mRNAs of diverse eukaryotic cells (Kozak, 1984 and 1986). Beginning from the ATG, the open reading frame was 5,214 bases encoding a protein of 205 kilodaltons.

4.4b Matrix homology plots of the Rb21-channel

The deduced sequence and size of the Rb21-channel showed similarity to those of reported Ca^{2+} and Na^{+} channels, leading to the hypothesis that the Rb21-channel may have 4-homologous domains such as voltage activated Ca^{2+} and Na^{+} channels. This hypothesis was examined by matrix homology plots that were designed to detect similar regions between two sequences by consecutively comparing parts of a sequence to the other sequence. For example, when rabbit α1S sequences plotted on x- and y-axes are compared to each other, the DHP receptor was found to consist of four homologous domains (Tanabe *et al.*, 1987).

The Rb21-channel sequence was compared with itself, the rabbit cardiac L-type Ca^{2+} channel, and the rat brain type I Na^{+} channel sequences. To increase the signal-to-noise ratio,


-91
GGGCTTAGCA
-1
AGTTCAGCCTGGTTAAGTCCAAGCTGAATTCCGGGCGCGCTGCGGTTACAGACTGTGGTTTTGTACCTGCCTCCCACAGCTAACTTCACC
1
90
ATGCTCAAAAGAAAGCAGAGTTCCAGGGTGGAAGCCCAGCCAGTAACTGACTTTGGTCCTGACGAATCTCTGTCAGACAATGCTGACATA
M L K R K Q S S R V E A Q P V T D F G P D E S L S D N A D I
$
IS1
CTCTGGATTAATAAGCCATGGGTGCACTCTCTGCTGCGCATCTGTGCCATCATCAGCGTCATCTCAGTGTGCATGAACACACCTATGACC
L W I N K P W V H S L L R I C A I I S V I S V C M N T P M T
IS2
TTCGAGCACTATCCTCCCCTTCAGTATGTGACCTTCACCCTGGACACTTTACTGATGTTTCTCTACACTGCAGAGATGATAGCAAAGATG
F E H Y P P L Q Y V T F T L D T L L M F L Y T A E M I A K M
IS3
CACATACGGGGAATTGTCAAGGGCGATAGCTCCTATGTGAAGGACCGCTGGTGTGTTTTTGATGGATTCATGGTCTTTTGCCTTTGGGTT
H I R G I V K G D S S Y V K D R W C V F D G F M V F C L W V
TCTCTCGTGTTACAGGTGTTTGAAATAGCTGACATAGTTGATCAGATGTCACCTTGGGGCATGCTGCGGATCCCACGGCCACTCATTATG
S L V L Q V F E I A D I V D Q M S P W G M L R I P R P L I M
IS4
ATCCGGGCTTTCAGGATTTATTTCCGATTCGAACTGCCAAGGACCCGAATTACAAACATTTTAAAGCGGTCAGGAGAACAAATATGGAGT
I R A F R I Y F R F E L P R T R I T N I L K R S G E Q I W S
IS5
630
GTCTCAATCTTCCTCCTCTTCTTCCTTCTTCTGTATGGGATTTTAGGAGTTCAGATGTTTGGAACATTTACCTACCACTGCGTAGTCAAT
V S I F L L F F L L L Y G I L G V Q M F G T F T Y H C V V N
#
GACACAAAGCCAGGGAATGTAACCTGGAATAGCTTAGCTATCCCAGATACGCACTGCTCGCCAGAGCTAGAAGAAGGCTATCAGTGCCCC
D T K P G N V T W N S L A I P D T H C S P E L E E G Y Q C P
#
CCAGGATTTAAATGCATGGACCTGGAAGACCTGGGACTTAGCAGGCAAGAGCTGGGCTACAGTGGCTTTAATGAGATAGGCACGAGTATA
P G F K C M D L E D L G L S R Q E L G Y S G F N E I G T S I
Ipore
TTCACAGTCTATGAGGCTTCATCTCAGGAAGGCTGGGTATTCCTCATGTACAGAGCAATCGACAGTTTCCCCCGTTGGCGTTCCTACTTC
F T V Y E A S S Q E G W V F L M Y R A I D S F P R W R S Y F
IS6
TACTTCATCACGCTGATTTTCTTCCTTGCCTGGCTTGTCAAGAATGTGTTCATTGCTGTCATCATTGAGACATTTGCAGAAATCAGAGTA
Y F I T L I F F L A W L V K N V F I A V I I E T F A E I R V
993
CAATTTCAACAAATGTGGGGAACTCGGAGCAGCACAACTTCCACTGCCACCACGCAGATGTTCCATGAAGATGCTGCCGGTGGCTGGCAG
Q F Q Q M W G T R S S T T S T A T T Q M F H E D A A G G W Q
CTGGTAGCTGTGGATGTCAACAAGCCCCAGGGACGTGCCCCAGCCTGCCTACAGAAAATGATGCGGTCATCAGTTTTCCACATGTTTATC
L V A V D V N K P Q G R A P A C L Q K M M R S S V F H M F I
IIS1
1260
CTGAGCATGGTGACTGTGGATGTAATAGTTGCTGCCAGCAACTACTACAAGGGAGAGAACTTCAGAAGGCAGTATGACGAGTTCTACCTT
L S M V T V D V I V A A S N Y Y K G E N F R R Q Y D E F Y L
&

```
1264----------IIS2----------                                    ------
GCAGAGGTGGCTTTTACAGTTCTTTTTGATCTGGAAGCACTTCTGAAGATATGGTGTTTGGGGTTTACTGGCTACATCAGCTCATCTCTC
 A  E  V  A  F  T  V  L  F  D  L  E  A  L  L  K  I  W  C  L  G  F  T  G  Y  I  S  S  S  L

----------IIS3----------                                    ----------
CACAAGTTTGAACTACTTCTGGTTATTGGGACAACTCTCCATGTATACCCTGATCTTTATCATTCTCAATTTACCTACTTCCAGGTTCTT
 H  K  F  E  L  L  L  V  I  G  T  T  L  H  V  Y  P  D  L  Y  H  S  Q  F  T  Y  F  Q  V  L

----------IIS4----------                                    ----------
CGGGTAGTCCGGCTTATTAAGATTTCCCCAGCATTGGAAGATTTTGTGTACAAGATATTTGGTCCCGGGAAAAAACTTGGAAGCTTGGTG
 R  V  V  R  L  I  K  I  S  P  A  L  E  D  F  V  Y  K  I  F  G  P  G  K  K  L  G  S  L  V

----------IIS5----------                                    ----------
GTGTTCACTGCCAGTCTCCTGATAGTTATGTCCGCCATCAGTTTGCAGATGTTCTGCTTCGTCGAAGAACTGGACAGATTCACCACATTT
 V  F  T  A  S  L  L  I  V  M  S  A  I  S  L  Q  M  F  C  F  V  E  E  L  D  R  F  T  T  F

----------IIpore----------                                   1710
CCAAGGGCATTTATGTCCATGTTCCAGATCCTCACCCAGGAAGGATGGGTGGATGTGATGGATCAGACTCTGAATGCTGTGGGGCATATG
 P  R  A  F  M  S  M  F  Q  I  L  T  Q  E  G  W  V  D  V  M  D  Q  T  L  N  A  V  G  H  M

          ----------IIS6----------
TGGGCTCCACTGGTTGCCATCTATTTCATCCTCTACCATCTCTTTGCAACGCTGATCCTCCTGAGTTTGTTTGTTGCTGTTATTTTGGAC
 W  A  P  L  V  A  I  Y  F  I  L  Y  H  L  F  A  T  L  I  L  L  S  L  F  V  A  V  I  L  D

------
AACTTAGAACTTGATGAAGATCTAAAGAAGCTCAAACAATTAAAACAAAGTGAAGCGAACGCAGATACCAAAGAAAAACTTCCTTTGCGC
 N  L  E  L  D  E  D  L  K  K  L  K  Q  L  K  Q  S  E  A  N  A  D  T  K  E  K  L  P  L  R

1984
TTGAGAATCTTCGAAAAATTCCCAAACAGACCGCAAATGGTGAAAATCTCAAAACTTCCTTCAGATTTTACAGTTCCTAAGATCAGGGAA
 L  R  I  F  E  K  F  P  N  R  P  Q  M  V  K  I  S  K  L  P  S  D  F  T  V  P  K  I  R  E

AGCTTCATGAAGCAGTTCATTGACCGCCAGCAACAGGACACCTGCTGTCTCTTCAGAATTCTCCCCTCTACCTCTTCCTCATCCTGTGAC
 S  F  M  K  Q  F  I  D  R  Q  Q  Q  D  T  C  C  L  F  R  I  L  P  S  T  S  S  S  S  C  D

AACCCCAAGCGGCCCACAGTTGAAGACAACAAATACATTGATCAAAAACTCCGCAAGTCTGTTTTCAGCATAAGGGCAAGGAACCTTCTG
 N  P  K  R  P  T  V  E  D  N  K  Y  I  D  Q  K  L  R  K  S  V  F  S  I  R  A  R  N  L  L
             $                    &                          *

GAAAAGGAGACCGCAGTCACAAAAATCTTAAGAGCTTGCACTCGACAGCGCATGCTGAGCGGATCATTCGAGGGGCAGCCAGCAAAGGAG
 E  K  E  T  A  V  T  K  I  L  R  A  C  T  R  Q  R  M  L  S  G  S  F  E  G  Q  P  A  K  E

AGGTCGATCCTCAGCGTGCAGCATCACATCCGCCAGGAGCGCAGGTCACTCAGACATGGATCCAACAGCCAGAGGATCAGCAGGGGCAAA
 R  S  I  L  S  V  Q  H  H  I  R  Q  E  R  R  S  L  R  H  G  S  N  S  Q  R  I  S  R  G  K
                                        *                       *

TCTCTTGAAACGTTAACTCAAGATCATTCCAATACGGTGCGCTACAGAAATGCACAAAGAGAAGACAGTGAAATAAAGATGATCCAGGAG
 S  L  E  T  L  T  Q  D  H  S  N  T  V  R  Y  R  N  A  Q  R  E  D  S  E  I  K  M  I  Q  E
                                    *

AAGAAGGAGCAAGCAGAGATGAAAAGGAAGGTGCAAGAAGAGGAGCTGCGAGAGAACCACCCATACTTTGACAAGCCTCTCTTCATCGTG
 K  K  E  Q  A  E  M  K  R  K  V  Q  E  E  E  L  R  E  N  H  P  Y  F  D  K  P  L  F  I  V

GGTCGAGAACACAGGTTCAGAAACTTCTGCCGCGTGGTGGTTCGAGCACGCTTCAATGCATCCAAAACAGACCCTGTCACGGGAGCTGTG
 G  R  E  H  R  F  R  N  F  C  R  V  V  V  R  A  R  F  N  A  S  K  T  D  P  V  T  G  A  V

2614                                    ----------IIIS1--------
AAAAATACAAAGTACCACCAACTTTATGATTTGCTGGGACTTGTCACCTACCTGGACTGGGTCATGATCACTGTAACCATCTGCTCTTGC
 K  N  T  K  Y  H  Q  L  Y  D  L  L  G  L  V  T  Y  L  D  W  V  M  I  T  V  T  I  C  S  C
```

```
----------                                                    ------------------------------
ATTTCTATGATGTTCGAATCCCCCTTCCGGAGAGTCATGCATGCACCCACTTTACAGATCGCTGAATATGTGTTTGTGATATTCATGAGC
 I  S  M  M  F  E  S  P  F  R  R  V  M  H  A  P  T  L  Q  I  A  E  Y  V  F  V  I  F  M  S

-----IIIS2----------------------------                        ------------------------
ATTGAGCTTAATCTGAAGATTATGGCAGATGGCTTGTTTTTCACTCCAACTGCTGTCATCAGGGACTTTGGTGGCGTCATGGACATATTT
 I  E  L  N  L  K  I  M  A  D  G  L  F  F  T  P  T  A  V  I  R  D  F  G  G  V  M  D  I  F

-----IIIS3----------------------------                        ------------------------------
ATCTATCTTGTGAGCTTGATATTTCTTTGTTGGATGCCTCAAAATGTGCCTGCTGAGTCAGGAGCCCAGCTCCTGATGGTTCTTCGGTGC
 I  Y  L  V  S  L  I  F  L  C  W  M  P  Q  N  V  P  A  E  S  G  A  Q  L  L  M  V  L  R  C

-----IIIS4-------------------------------------                 ---------------
CTAAGACCTCTTCGGATATTCAAACTGGTGCCACAAATGAGGAAAGTTGTTCGAGAACTTTTCAGTGGTTTCAAGGAAATATTTTTGGTC
 L  R  P  L  R  I  F  K  L  V  P  Q  M  R  K  V  V  R  E  L  F  S  G  F  K  E  I  F  L  V

----------------------IIIS5---------------------------------------                      3150
TCCATTCTGTTGCTGACACTAATGCTTGTTTTTGCAAGCTTTGGTGTTCAGCTCTTTGCCGGAAAGCTAGCCAAGTGCAATGACCCCAAC
 S  I  L  L  L  T  L  M  L  V  F  A  S  F  G  V  Q  L  F  A  G  K  L  A  K  C  N  D  P  N


ATTATCAGAAGGGAAGACTGTAACGGCATCTTCAGAATTAATGTAAGTGTGTCCAAGAACTTAAATTTAAAACTAAGACCTGGAGAGAAA
 I  I  R  R  E  D  C  N  G  I  F  R  I  N  V  S  V  S  K  N  L  N  L  K  L  R  P  G  E  K
                                          #

                                                  -------------------------------IIIpore------
AAACCTGGATTTTGGGTGCCCCGTGTTTGGGCAAATCCTCGTAACTTTAACTTTGACAATGTGGGAAATGCTATGCTGGCATTGTTTGAA
 K  P  G  F  W  V  P  R  V  W  A  N  P  R  N  F  N  F  D  N  V  G  N  A  M  L  A  L  F  E

-----------------------------------                 --------------------------------
GTTCTGTCCTTGAAAGGCTGGGTAGAAGTGAGAGATGTCATTATTCATCGTGTGGGGCCGATCCATGGAATCTATATTCATGTTTTCGTA
 V  L  S  L  K  G  W  V  E  V  R  D  V  I  I  H  R  V  G  P  I  H  G  I  Y  I  H  V  F  V

--------IIIS6-----------------------------------------------
TTCCTGGGTTGCATGATTGGACTGACTCTTTTTGTCGGTGTAGTTATTGCTAACTTCAATGAAAACAAGGGGACAGCCCTGCTGACGGTA
 F  L  G  C  M  I  G  L  T  L  F  V  G  V  V  I  A  N  F  N  E  N  K  G  T  A  L  L  T  V


GATCAGAGAAGATGGGAAGATCTCAAGAGCAGATTGAAGATCGCACAGCCTCTTCATCTCCCACCTCGGCCGGATAATGATGGATTTAGA
 D  Q  R  R  W  E  D  L  K  S  R  L  K  I  A  Q  P  L  H  L  P  P  R  P  D  N  D  G  F  R

                                  ------------------------------------IVS1-----------------------
GCTAAAATGTATGACATAACCCAGCATCCGTTTTTTAAAAGGACAATTGCGTTGCTGGTTCTGGCCCAGTCTGTGTTGCTATCTGTCAAG
 A  K  M  Y  D  I  T  Q  H  P  F  F  K  R  T  I  A  L  L  V  L  A  Q  S  V  L  L  S  V  K

------                                                ----------------------------------IVS2------------
TGGGATGTTGAGGATCCTGTGACAGTTCCTTTGGCAACCATGTCGGTCGTGTTCACCTTCATCTTTGTCTTAGAGGTTACAATGAAGATC
 W  D  V  E  D  P  V  T  V  P  L  A  T  M  S  V  V  F  T  F  I  F  V  L  E  V  T  M  K  I

---------------                        ----------------------------------IVS3--------------------
ATAGCAATGTCACCAGCTGGTTTCTGGCAAAGCAGAAGAAACCGATATGATCTCTTGGTGACGTCTCTTGGTGTTGTGTGGGTGGTGCTC
 I  A  M  S  P  A  G  F  W  Q  S  R  R  N  R  Y  D  L  L  V  T  S  L  G  V  V  W  V  V  L
                              *

---                                                   --------------------------------IVS4-----------
CATTTTGCTCTGCTGAATGCATACACCTACATGATGGGAGCCTGTGTGATTGTCTTTAGATTTTTCTCCATCTGTGGAAAGCATGTGACA
 H  F  A  L  L  N  A  Y  T  Y  M  M  G  A  C  V  I  V  F  R  F  F  S  I  C  G  K  H  V  T

------------------                      ------------------------------------IVS5-----------
TTAAAGATGCTCCTTTTGACTGTGGTTGTCAGCATGTACAAGAGCTTCTTTATCATCGTAGGAATGTTTCTCTTGCTGCTGTGCTATGCC
 L  K  M  L  L  L  T  V  V  V  S  M  Y  K  S  F  F  I  I  V  G  M  F  L  L  L  L  C  Y  A

------------------------------                                ---------------------------
TTTGCTGGGGTGGTTCTGTTTGGCACTGTAAAGTATGGAGAGAACATTAACAGGCATGCCAATTTTTCTTCAGCTGGCAAAGCCATTACT
 F  A  G  V  V  L  F  G  T  V  K  Y  G  E  N  I  N  R  H  A  N  F  S  S  A  G  K  A  I  T
                                                          #
```

```
----------IVpore--------------------------------
GTATTGTTCCGAATTGTCACAGGGGAAGACTGGAATAAGATTATGCATGATTGTATGGTTCAGCCTCCATTTTGTACTCCTGATGAATTT
 V  L  F  R  I  V  T  G  E  D  W  N  K  I  M  H  D  C  M  V  Q  P  P  F  C  T  P  D  E  F

                                    ----------------------------------------IVS6-------------
ACATATTGGGCAACAGACTGTGGCAATTATGCAGGGGCACTTATGTACTTCTGCTCATTCTATGTCATCATCGCCTACATCATGCTGAAT
 T  Y  W  A  T  D  C  G  N  Y  A  G  A  L  M  Y  F  C  S  F  Y  V  I  I  A  Y  I  M  L  N

------------------------------
CTACTTGTAGCCATAATTGTGGAGAATTTCTCTTTGTTTTATTCCACTGAAGAGGACCAGCTTTTGAGTTACAATGATCTTCGCCATTTT
 L  L  V  A  I  I  V  E  N  F  S  L  F  Y  S  T  E  E  D  Q  L  L  S  Y  N  D  L  R  H  F

CAAATCATATGGAATATGGTAGATGATAACGAGAGGTGTGATCCCACCTTCCGAGTGAAGTTCCTGCTACGGCTACTGCGTGGGAGGCTA
 Q  I  I  W  N  M  V  D  D  N  E  R  C  D  P  T  F  R  V  K  F  L  L  R  L  L  R  G  R  L
                                                *

GAAGTGGACCTTGATAAAGACAAGCTCCTGTTTAAGCACATGTGCTATGAGATGGAGAGGCTGCACAATGGTGGTGATGTCACCTTCCAT
 E  V  D  L  D  K  D  K  L  L  F  K  H  M  C  Y  E  M  E  R  L  H  N  G  G  D  V  T  F  H

GATGTCTTGAGCATGCTGTCCTACCGCTCTGTAGACATCAGGAAGAGCCTGCAGCTAGAGGAACTGCTGGCAAGGGAGCAGCTGGAGTAC
 D  V  L  S  M  L  S  Y  R  S  V  D  I  R  K  S  L  Q  L  E  E  L  L  A  R  E  Q  L  E  Y
                   *

ACCATAGAGGAGGAGGTGGCTAAGCAGACTATTCGCATGTGGCTGAAGAAGTGCTTAAAACGCATCCGGGCTAAACAGCAGCAGTCATGC
 T  I  E  E  E  V  A  K  Q  T  I  R  M  W  L  K  K  C  L  K  R  I  R  A  K  Q  Q  Q  S  C
                            *

AGCATCATCCACAGCCTGAGAGAGAGCCAGCAGCAAGAGTTGAGCCGATTCCTGAATCCTCCCAGCATTGAGACCACCCAACCAAGCGAA
 S  I  I  H  S  L  R  E  S  Q  Q  Q  E  L  S  R  F  L  N  P  P  S  I  E  T  T  Q  P  S  E
               *

GATACCAACGCCAACAGCCAGGACCACAATACGCAACCTGAGTCAAGCAGTCAGCAGCAGCTCTTGAGCCCTACTCTGTCAGACAGAGGA
 D  T  N  A  N  S  Q  D  H  N  T  Q  P  E  S  S  S  Q  Q  Q  L  L  S  P  T  L  S  D  R  G
                                                                  *

GGAAGTCGACAGGATGCAGCAGATACTGGGAAACCCCAAAGAAAGATTGGGCAATGGCGTCTGCCCTCAGCTCCCAAACCAATAAGCCAT
 G  S  R  Q  D  A  A  D  T  G  K  P  Q  R  K  I  G  Q  W  R  L  P  S  A  P  K  P  I  S  H
                        *

TCTGTGTCTTCGGTTAACCTACGGTTTGGAGGAAGGACAACAATGAAGTCTGTGGTGTGCAAGATGAACCCCATGCCAGACACAGCTTCC
 S  V  S  S  V  N  L  R  F  G  G  R  T  T  M  K  S  V  V  C  K  M  N  P  M  P  D  T  A  S
                                       *

                                                                                        5220
TGTGGCTCCGAAGTTAAAAAGTGGTGGACCAGACAGCTGACTGTGGAGAGTGACGAGAGTGGAGATGACCTCCTAGATATCTAGAGGGGA
 C  G  S  E  V  K  K  W  W  T  R  Q  L  T  V  E  S  D  E  S  G  D  D  L  L  D  I  @
                                                                              (1737)

AGCAAAGCATAGGGATTTATCGGGTGAAAACCACTGCCTAATCTCTTGTTTTATCTCTGAGAGAGCAAACTGTAGTTGCCAAACCCTAAA
TTTCACCCGAGCATGGCATTTTCAGAATTAACTTTTGTTCATTTATTGGAATGGTCCCTCAAAGAGTATGAAGTTTCAGATTACTCTCAT
GTATTAAAGAAAATCTGGGGCTTGGGATGTAAGTCAGTGAAAGACCACTTGTCCAACCTATGAAAACCCGTGTGTTTAAAACCCAGAAAT
ACAAGAAAAGAAGGAAAATATGTATAATGCAGTATTGATAATATTAGAAGCTGCATCAATAGTAGATGTGTATTAAATATTCATGTATTC
TGTCTGTGTAAGATAAGCTGTACTCACCTGCACTCCTGTATTTTGTTACTACTCCTCTCAAAGAGTGCTAAGCCCATACTGGCCCATATT
GATAGTACAGGAGTTGTAGCTCTAGTTCACCTTTTTTTCCCATTTCCAATAGAAATACTTTCCTTGGGAGAAATTATTTATGATTGATCT
GAAAAGGTCAATCATGCTAAAATGACAGCAGCTTCAAAACTACAGATTCTGGATTTTTAAAAACATTCCTTTTCTCATATTATTTAAAAA
ATATGCCTGATGTTAAAAAAATTAGACAATTTGATTTTTTATTTCAAGTACTCTAATTGAACTGTGGCCTGCTTCCAAGAAAGTTATATA
TGTAGGTCCCATGTAAAGGAACTGGCTATAGATATAATTGGTATATAACAAACCTGTAGGTATATTATTCATGGACTTGATGAGACAAAA
ATATTTTGAAATCTTTAAAAACCCTTTAAATAACTGAGTTTATAAAAAAAGAATAAAAGTGTTGGAGTCAACATAGCTAAAAGTTTACAC
TTGAGACTTATTGTGGACTTCATGGCTTATCCTTGCTGTATCCATTTTGCTATGAATCAGCAGGAACATTATGGGTTGGCTTTAATACAC
CATTACTATGTAGATTGAAAATAATTTCCCTGCTTCCAGAATTATCTATTGAAATAATAACAGAAGGGTGCCAAAACTTCACTCTAAACC
CCATTGGGAATACCACAACATAATTATATTTTCCCACACACCAATGCTCCAGGTTCTCTGGGAAAAGTGGAAATATAAAATTATTGGAAA
GTATTGATGGGCCCTGGCTGGGCAGAGTCTCCCCTGAGTTGTCATTATCAATTGCATATTTGATGATCAGATTATTTTAAGAGATTGAGT
GTGTAAAAATTCCCTGATGCCTAGGTTGCTTTATGATATCCTTTTGTCTATTTAACCTTCTTCAAATAGCCAAACATTGTGTCCTTCCTA
CACAGAAGATGTGATATAATGTGCCCATCTACTGCTTTTATGATTACTTGTGACTATTAGATAATAGGCAATTCAAGTTCATGCCGCCAA
GGGGCCTTTTTATTGGGTTTTATGTTTGGTCAGAATTTGATTTTTGGATATAAAAGCCGG
                                                                  6720
```

Figure 11. Full-length cDNA and translated amino acid sequence of the Rb21-channel, a putative ion channel like Ca^{2+} or Na^{+} channel. The full-length cDNA is 6820 bases. The open reading frame of Rb21-cDNA is translated into amino acids using a DNASIS program (Hitachi, version 7.07). The open reading frame of the cloned cDNA is 5,214 bases and the molecular weight of the deduced amino acid sequence is about 205 kdaltons. The putative membrane spanning portions are hydrophobic regions detected by a hydropathicity analysis (refer to figure 12). Putative membrane spanning segments are indicated above the translation. Potential consensus sequences related to important physiological functions are analyzed by Prosis program and marked as follows: 4 N-glycosylation sites (#), 2 cAMP-dependent protein kinase A phosphorylation sites ($), 2 tyrosine kinase phosphorylation sites (&), and 12 protein kinase C phosphorylation sites (*). @ repesents a stop codon.

poorly conserved cytoplasmic loops were not used for this analysis. In figure 12A, the Rb21-channel sequence (y-axis) was compared with itself (x-axis). The matrix homology plot contains one diagonal line plus three parallel lines. The diagonal line represents the identity of sequences used in both x- and y-axes. The diagonal and three parallel lines in both upper and lower side of the diagonal line indicate that the Rb21-channel contains four domains, which share sequence homology one another. To check that the Rb21-channel is similar to reported Ca^{2+} and Na^{+} channels in terms of sequence homology and four domain structure, matrix homology plots were performed between the sequence of the Rb21-channel and those of the rabbit cardiac L-type Ca^{2+} channel and the rat brain type I Na^{+} channel (figure 12B and C). Patterns detected by matrix homology plots were similar to that of the Rb21-channel with itself, but there appeared four separated lines instead of a diagonal line. Four disconnected lines in the diagonal position and neighboring three parallel lines supported that the Rb21 channel shares not only sequence homology, but also a four domain structure with Ca^{2+} and Na^{+} channels.

4.4c Hydropathy analysis of the Rb21-channel

Hydropathy analysis was performed to predict the putative membrane spanning segments and loop portions of the Rb21-channel (figure 13). The analyzed pattern of hydrophobic and hydrophilic portions was compared to those of previously cloned Ca^{2+} and Na^{+} channel sequences.

Hydropathy analysis indicated that the Rb21-channel has 4 repeats (also called domains) which are connected by hydrophilic cytoplasmic loops. Each repeat contains 6

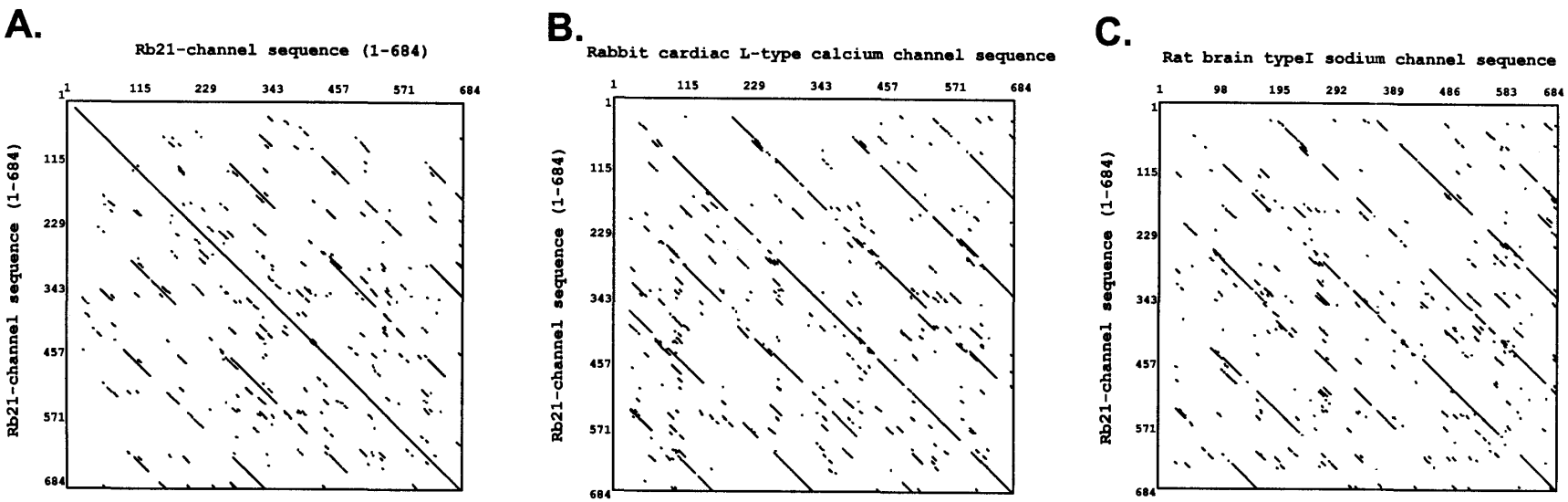


Figure 12. Matrix homology plots of the Rb21-channel with itself, a rabbit cardiac L-type calcium channel, and a rat brain type I sodium channel sequence. The membrane spanning amino acid sequence of the Rb21-channel (y-axis) is compared with itself in the x-axis using the Needleman and Wunsch algorithm (1970) in the WDNASIS program (Hitachi) (A). The matrix homology plot is performed using following parameters: the checking size is 30 amino acids; the matching size is 15 amino acids. More than 15 equivalent amino acids (50 %) among 30 amino acids of a checking window would produce a dot, representing homology. The sequence of the Rb21-channel is also compared with those of the rabbit cardiac L-type calcium channel α1C (B) and the rat brain type I sodium channel (C).

membrane spanning segments, a pore loop, and external and internal connecting loops. S1, S2, S3, S5, S6 of each domain are detected with distinctive hydrophobic indices, but S4 and pore regions have smaller indices. The reason that S4 and pores have low indices might be due to their partial exposure to aqueous environment (Yang *et al.*, 1996). These analyzed structural properties of the Rb21-channel were similar to those of reported Ca^{2+} and Na^{+} channels, suggesting that the Rb21-channel may have a similar overall structure, and function as a voltage-activated cation channel.

4.4d Alignment of the Rb21-channel with Ca^{2+} and Na^{+} channels

To compare the amino acid sequence of the Rb21-channel with those of reported Ca^{2+} and Na^{+} channels in detail, putative membrane spanning sequences of the Rb21-channel, *C. elegans* putative ion channel (C27f2), six Ca^{2+} channels, and eight Na^{+} channels were aligned in figure 14. Hydrophilic loop portions of the channel sequences were not conserved in size and homology so that only hydrophobic putative membrane spanning portions were aligned. Each residue of the Rb21-channel could be compared to that in corresponding positions of reported Ca^{2+} and Na^{+} channels. Based on their homology, levels of conservation were marked by different symbols. The probability that a same residue is found in 14 different channel sequences is 20^{-14} (= 6.1×10^{-19}). Also, amino acid residues in the alignment were marked with different colors according to their hydrophobicity and charge, revealing that residues of Rb21 channel have similar structural properties with corresponding residues of reported Ca^{2+} and Na^{+} channels. The alignment also showed that Rb21-channel shared conserved amino acid residues, especially in S4 segments and pore loops, with reported Ca^{2+}

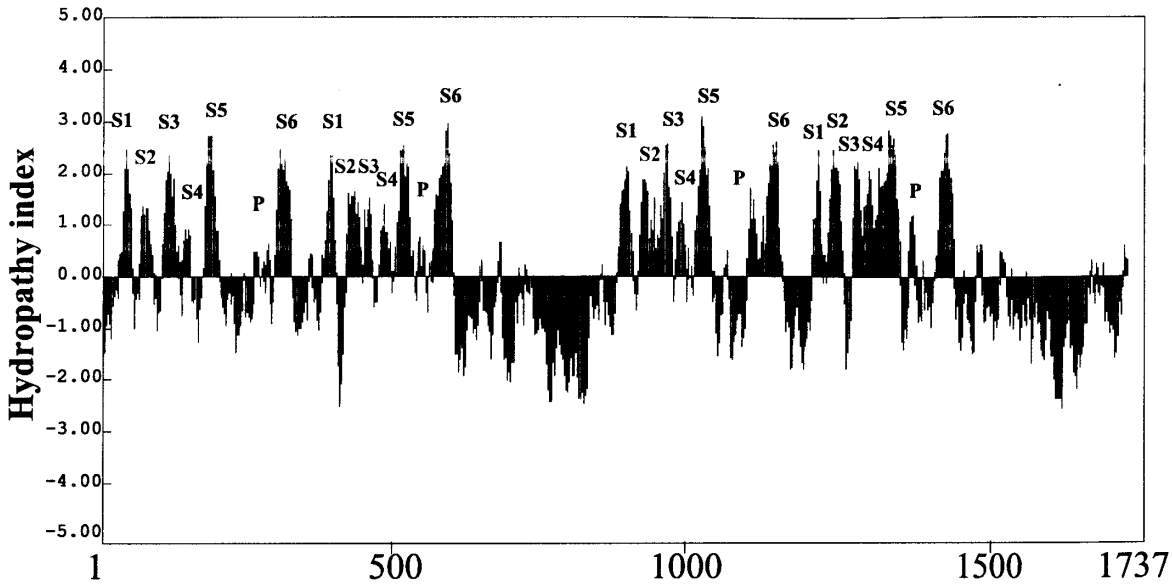
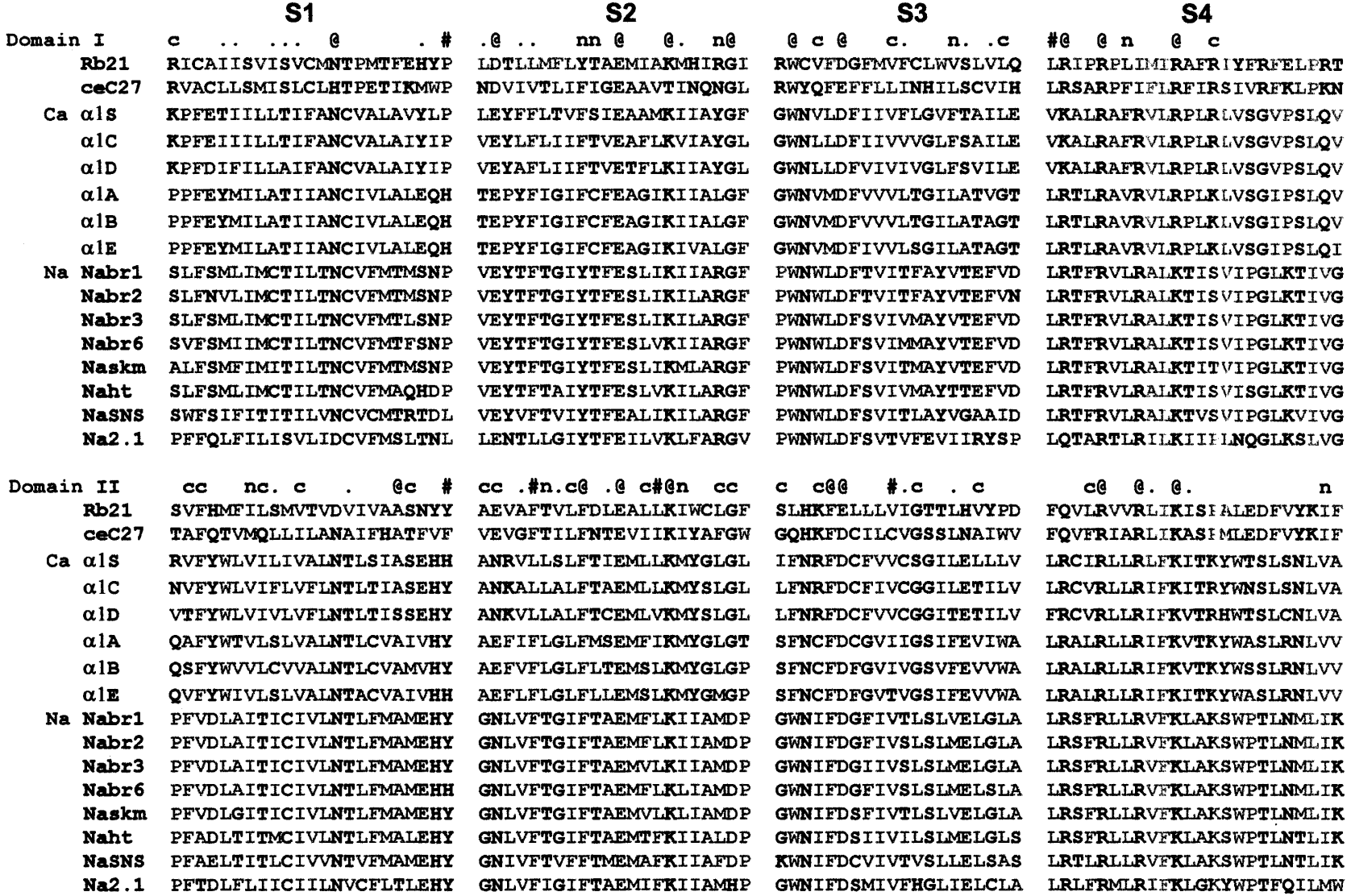
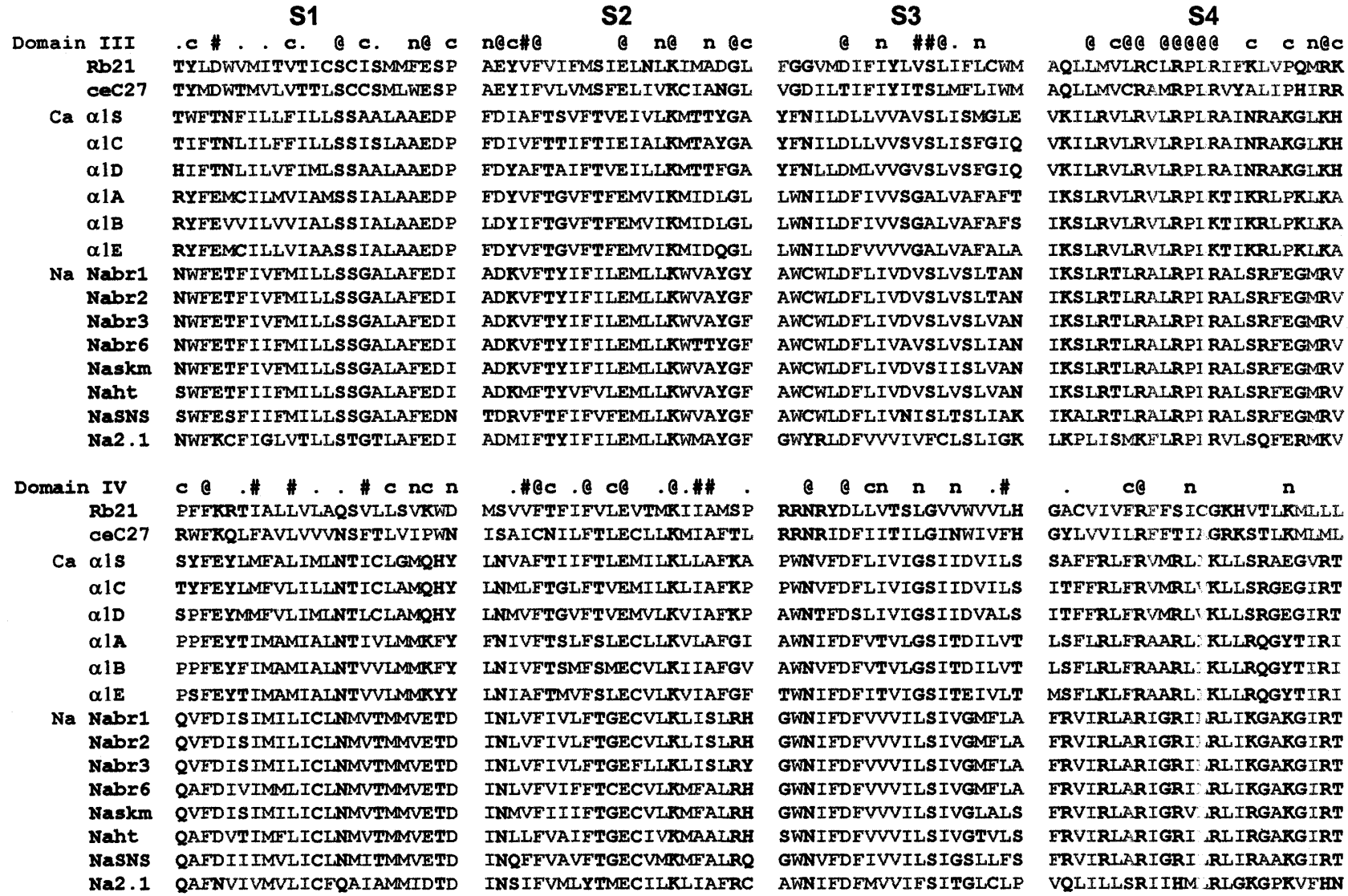


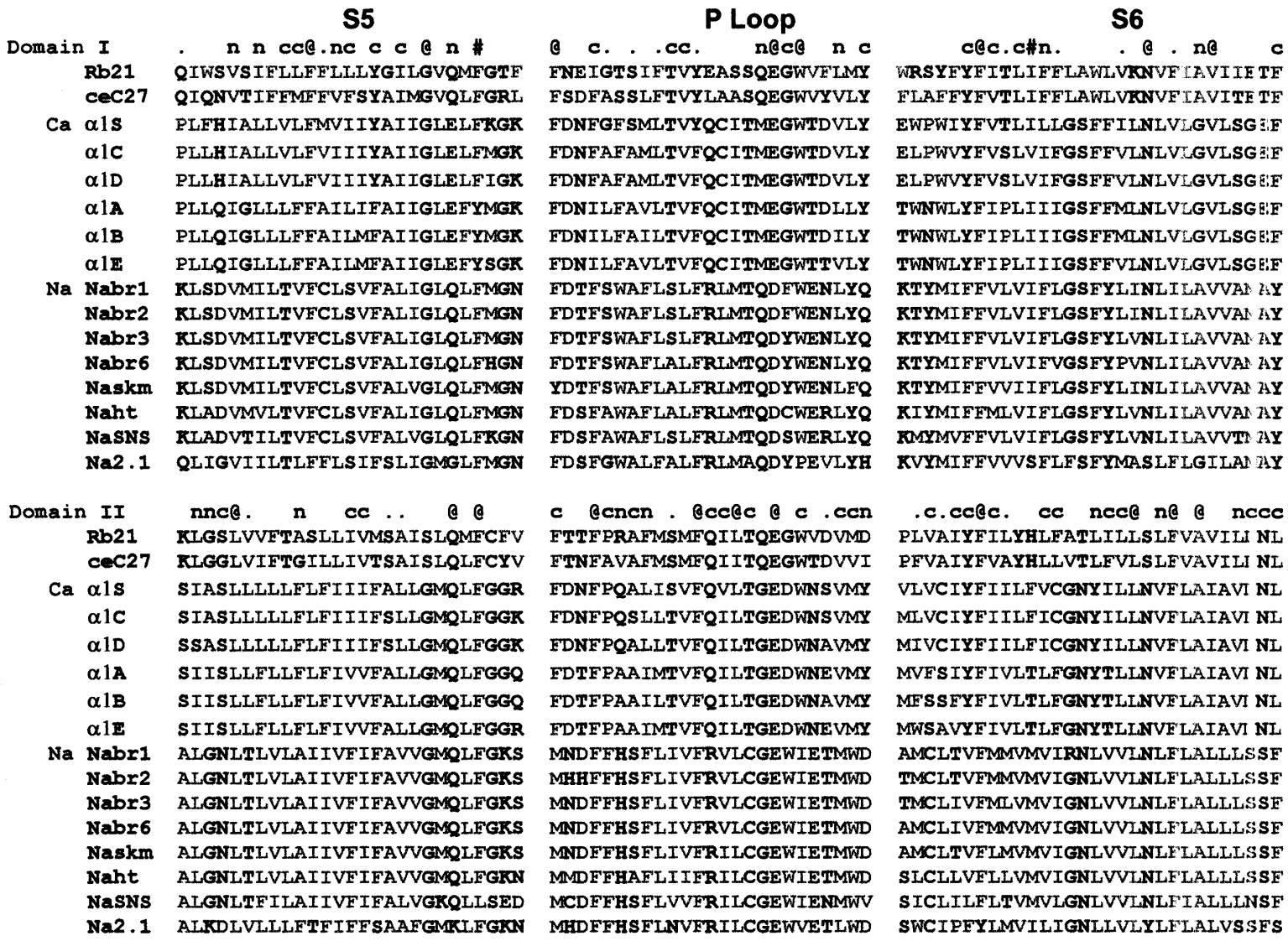
Figure 13. Hydropathicity plot of the Rb-21 channel. The hydropathicity analysis was performed using the algorithm of Kyte and Doolittle (1982) in a WDNASIS program (Hitachi). The amino acid residues of the Rb21-channel are plotted along the x-axis, and the calculated hydropathy indexes based on a running average of 14 amino acids are plotted along the y-axis. The hydrophobicity profile of the Rb21-channel were compared with those of reported Ca^{2+} and Na^{+} channels. Putative membrane spanning segments of each domain detected as hydrophobic regions are labeled as S1, S2, S3, S4, S5, P (pore), and S6.

and Na^+ channels . Compared with the S4 segments of the Na^+ and Ca^{2+} channels, however, the S4 segments of the Rb21 channel contained less positively charged amino acids (arginine, lysine, or histidine). These different characteristics of the voltage sensor might be related to the distinctive function of the Rb21-channel (Stümer *et al.*, 1989; Yang *et al.*, 1996). For example, the modified voltage sensor of the Rb21 channel may cause unique activation and inactivation behaviors such as low VACCs.

The negatively-charged amino acids (glutamic acids, E) in the pores of domain I, II, III, and IV (EEEE) of cloned mammalian Ca^{2+} channels have been reported to determine Ca^{2+} selectivity for high VACCs (Heinemann *et al.*, 1992; Tang *et al.*, 1993; Yang *et al.*, 1993; Parent and Gopalakrishnan, 1995). In an analogous position in voltage-activated Na^+ channels, these residues are DEKA: negatively-charged amino acids (aspartic acid,D; glutamic acid, E) in the corresponing regions of the domain I and II; a positive residue (lysine, K) in domain III; and a neutral residue (alanine, A) in domain IV. It was proposed that the differently charged residues of domain III and IV pores might distinguish the ion selectivity of Ca^{2+} and Na^+ channels. Heinemann and his colleagues tested this hypothesis by examining ion permeation properties of point-mutated Na^+ channels in which K of domain III pore (DEEA), A of domain IV pore (DEKE), or both (DEEE) were replaced with E. The DEEA and DEEE channels showed a decrement of Na^+ selectivity and permeability to Ba^{2+} and Ca^{2+}, supporting the hypothesis that the positive and neurtral residues in domain III and IV make ion selectivity of Na^+ channels different from Ca^{2+} channels. This idea was examined using similarly point-mutated Ca^{2+} channels (Tang *et al.*, 1993; Yang *et al.*, 1993; Parent and Gopalakrishnan, 1995). A site-directed point mutation of E in domain III pore


S1
S2
S3
S4
Domain I
c .. ... @ . #
.@ .. nn @ @. n@
@ c @ c. n. .c
#@ @ n @ c
Rb21 RICAIISVISVCMNTPMTFEHYP LDTLLMFLYTAEMIAKMHIRGI RWCVFDGFMVFCLWVSLVLQ LRIPRPLIMIRAFRIYFRFELFRT
ceC27 RVACLLSMISLCLHTPETIKMWP NDVIVTLIFIGEAAVTINQNGL RWYQFEFFLLINHILSCVIH LRSARPFIFLRFIRSIVRFKLPKN
Ca α1S KPFETIILLTIFANCVALAVYLP LEYFFLTVFSIEAAMKIIAYGF GWNVLDFIIVFLGVFTAILE VKALRAFRVLRPLRLVSGVPSLQV
α1C KPFEIIILLTIFANCVALAIYIP VEYLFLIIFTVEAFLKVIAYGL GWNLLDFIIVVVGLFSAILE VKALRAFRVLRPLRLVSGVPSLQV
α1D KPFDIFILLAIFANCVALAIYIP VEYAFLIIFTVETFLKIIAYGL GWNLLDFVIVIVGLFSVILE VKALRAFRVLRPLRLVSGVPSLQV
α1A PPFEYMILATIIANCIVLALEQH TEPYFIGIFCFEAGIKIIALGF GWNVMDFVVVLTGILATVGT LRTLRAVRVLRPLKLVSGIPSLQV
α1B PPFEYMILATIIANCIVLALEQH TEPYFIGIFCFEAGIKIIALGF GWNVMDFVVVLTGILATAGT LRTLRAVRVLRPLKLVSGIPSLQV
α1E PPFEYMILATIIANCIVLALEQH TEPYFIGIFCFEAGIKIVALGF GWNVMDFIVVLSGILATAGT LRTLRAVRVLRPLKLVSGIPSLQI
Na Nabr1 SLFSMLIMCTILTNCVFMTMSNP VEYTFTGIYTFESLIKIIARGF PWNWLDFTVITFAYVTEFVD LRTFRVLRALKTISVIPGLKTIVG
Nabr2 SLFNVLIMCTILTNCVFMTMSNP VEYTFTGIYTFESLIKILARGF PWNWLDFTVITFAYVTEFVN LRTFRVLRALKTISVIPGLKTIVG
Nabr3 SLFSMLIMCTILTNCVFMTLSNP VEYTFTGIYTFESLIKILARGF PWNWLDFSVIVMAYVTEFVD LRTFRVLRALKTISVIPGLKTIVG
Nabr6 SVFSMIIMCTILTNCVFMTFSNP VEYTFTGIYTFESLVKIIARGF PWNWLDFSVIMMAYVTEFVD LRTFRVLRALKTISVIPGLKTIVG
Naskm ALFSMFIMITILTNCVFMTMSNP VEYTFTGIYTFESLIKMLARGF PWNWLDFSVITMAYVTEFVD LRTFRVLRAIKTITVIPGLKTIVG
Naht SLFSMLIMCTILTNCVFMAQHDP VEYTFTAIYTFESLVKILARGF PWNWLDFSVIVMAYTTEFVD LRTFRVLRALKTISVISGLKTIVG
NaSNS SWFSIFITITILVNCVCMTRTDL VEYVFTVIYTFEALIKILARGF PWNWLDFSVITLAYVGAAID LRTFRVLRALKTVSVIPGLKVIVG
Na2.1 PFFQLFILISVLIDCVFMSLTNL LENTLLGIYTFEILVKLFARGV PWNWLDFSVTVFEVIIRYSP LQTARTLRILKIIRHLNQGLKSLVG
Domain II
cc nc. c . @c # cc .#n.c@ .@ c#@n cc c c@@ #.c . c c@ @. @. n
Rb21 SVFHMFILSMVTVDVIVAASNYY AEVAFTVLFDLEALLKIWCLGF SLHKFELLLVIGTTLHVYPD FQVLRVVRLIKISRALEDFVYKIF
ceC27 TAFQTVMQLLILANAIFHATFVF VEVGFTILFNTEVIIKIYAFGW GQHKFDCILCVGSSLNAIWV FQVFRIARLIKASRMLEDFVYKIF
Ca α1S RVFYWLVILIVALNTLSIASEHH ANRVLLSLFTIEMLLKMYGLGL IFNRFDCFVVCSGILELLLV LRCIRLLRLFKITKYWTSLSNLVA
α1C NVFYWLVIFLVFLNTLTIASEHY ANKALLALFTAEMLLKMYSLGL LFNRFDCFIVCGGILETILV LRCVRLLRIFKITRYWNSLSNLVA
α1D VTFYWLVIVLVFLNTLTISSEHY ANKVLLALFTCEMLVKMYSLGL LFNRFDCFVVCGGITETILV FRCVRLLRIFKVTRHWTSLCNLVA
α1A QAFYWTVLSLVALNTLCVAIVHY AEFIFLGLFMSEMFIKMYGLGT SFNCFDCGVIIGSIFEVIWA LRALRLLRIFKVTKYWASLRNLVV
α1B QSFYWVVLCVVALNTLCVAMVHY AEFVFLGLFLTEMSLKMYGLGP SFNCFDFGVIVGSVFEVVWA LRALRLLRIFKVTKYWSSLRNLVV
α1E QVFYWIVLSLVALNTACVAIVHH AEFLFLGLFLLEMSLKMYGMGP SFNCFDFGVTVGSIFEVVWA LRALRLLRIFKITKYWASLRNLVV
Na Nabr1 PFVDLAITICIVLNTLFMAMEHY GNLVFTGIFTAEMFLKIIAMDP GWNIFDGFIVTLSLVELGLA LRSFRLLRVFKLAKSWPTLNMLIK
Nabr2 PFVDLAITICIVLNTLFMAMEHY GNLVFTGIFTAEMFLKIIAMDP GWNIFDGFIVSLSLMELGLA LRSFRLLRVFKLAKSWPTLNMLIK
Nabr3 PFVDLAITICIVLNTLFMAMEHY GNLVFTGIFTAEMVLKIIAMDP GWNIFDGIIVSLSLMELGLA LRSFRLLRVFKLAKSWPTLNMLIK
Nabr6 PFVDLAITICIVLNTLFMAMEHH GNLVFTGIFTAEMFLKLIAMDP GWNIFDGFIVSLSLMELSLA LRSFRLLRVFKLAKSWPTLNMLIK
Naskm PFVDLGITICIVLNTLFMAMEHY GNLVFTGIFTAEMVLKLIAMDP GWNIFDSFIVTLSLVELGLA LRSFRLLRVFKLAKSWPTLNMLIK
Naht PFADLTITMCIVLNTLFMALEHY GNLVFTGIFTAEMTFKIIALDP GWNIFDSIIVILSLMELGLS LRSFRLLRVFKLAKSWPTLNTLIK
NaSNS PFAELTITLCIVVNTVFMAMEHY GNIVFTVFFTMEMAFKIIAFDP KWNIFDCVIVTVSLLELSAS LRTLRLLRVFKLAKSWPTLNTLIK
Na2.1 PFTDLFLIICIILNVCFLTLEHY GNLVFIGIFTAEMIFKIIAMHP GWNIFDSMIVFHGLIELCLA LRLFRMLRIFKLGKYWPTFQILMW


S1
S2
S3
S4
Domain III .c # . . c. @ c. n@ c n@c#@ @ n@ n @c @ n ##@. n @ c@@ @@@@@ c c n@c
Rb21 TYLDWVMITVTICSCISMMFESP AEYVFVIFMSIELNLKIMADGL FGGVMDIFIYLVSLIFLCWM AQLLMVLRCLRPLRIFKLVPQMRK
ceC27 TYMDWTMVLVTTLSCCSMLWESP AEYIFVLVMSFELIVKCIANGL VGDILTIFIYITSLMFLIWM AQLLMVCRAMRPLRVYALIPHIRR
Ca α1S TWFTNFILLFILLSSAALAAEDP FDIAFTSVFTVEIVLKMTTYGA YFNILDLLVVAVSLISMGLE VKILRVLRVLRPLRAINRAKGLKH
α1C TIFTNLILFFILLSSISLAAEDP FDIVFTTIFTIEIALKMTAYGA YFNILDLLVVSVSLISFGIQ VKILRVLRVLRPLRAINRAKGLKH
α1D HIFTNLILVFIMLSSAALAAEDP FDYAFTAIFTVEILLKMTTFGA YFNLLDMLVVGVSLVSFGIQ VKILRVLRVLRPLRAINRAKGLKH
α1A RYFEMCILMVIAMSSIALAAEDP FDYVFTGVFTFEMVIKMIDLGL LWNILDFIVVSGALVAFAFT IKSLRVLRVLRPLKTIKRLPKLKA
α1B RYFEVVILVVIALSSIALAAEDP LDYIFTGVFTFEMVIKMIDLGL LWNILDFIVVSGALVAFAFS IKSLRVLRVLRPLKTIKRLPKLKA
α1E RYFEMCILLVIAASSIALAAEDP FDYVFTGVFTFEMVIKMIDQGL LWNILDFVVVVGALVAFALA IKSLRVLRVLRPLKTIKRLPKLKA
Na Nabr1 NWFETFIVFMILLSSGALAFEDI ADKVFTYIFILEMLLKWVAYGY AWCWLDFLIVDVSLVSLTAN IKSLRTLRALRPLRALSRFEGMRV
Nabr2 NWFETFIVFMILLSSGALAFEDI ADKVFTYIFILEMLLKWVAYGF AWCWLDFLIVDVSLVSLTAN IKSLRTLRALRPLRALSRFEGMRV
Nabr3 NWFETFIVFMILLSSGALAFEDI ADKVFTYIFILEMLLKWVAYGF AWCWLDFLIVDVSLVSLVAN IKSLRTLRALRPLRALSRFEGMRV
Nabr6 NWFETFIIFMILLSSGALAFEDI ADKVFTYIFILEMLLKWTTYGF AWCWLDFLIVAVSLVSLIAN IKSLRTLRALRPLRALSRFEGMRV
Naskm NWFETFIVFMILLSSGALAFEDI ADKVFTYIFILEMLLKWVAYGF AWCWLDFLIVDVSIISLVAN IKSLRTLRALRPLRALSRFEGMRV
Naht SWFETFIIFMILLSSGALAFEDI ADKMFTYVFVLEMLLKWVAYGF AWCWLDFLIVDVSLVSLVAN IKSLRTLRALRPLRALSRFEGMRV
NaSNS SWFESFIIFMILLSSGALAFEDN TDRVFTFIFVFEMLLKWVAYGF AWCWLDFLIVNISLTSLIAK IKALRTLRALRPLRALSRFEGMRV
Na2.1 NWFKCFIGLVTLLSTGTLAFEDI ADMIFTYIFILEMLLKWMAYGF GWYRLDFVVVIVFCLSLIGK LKPLISMKFLRPLRVLSQFERMKV
Domain IV c @ .# # . . # c nc n .#@c .@ c@ .@.## . @ @ cn n n .# . c@ n n
Rb21 PFFKRTIALLVLAQSVLLSVKWD MSVVFTFIFVLEVTMKIIAMSP RRNRYDLLVTSLGVVWVVLH GACVIVFRFFSICGKHVTLKMLLL
ceC27 RWFKQLFAVLVVVNSFTLVIPWN ISAICNILFTLECLLKMIAFTL RRNRIDFIITILGINWIVFH GYLVVILRFFTIAGRKSTLKMLML
Ca α1S SYFEYLMFALIMLNTICLGMQHY LNVAFTIIFTLEMILKLLAFKA PWNVFDFLIVIGSIIDVILS SAFFRLFRVMRLLKLLSRAEGVRT
α1C TYFEYLMFVLILLNTICLAMQHY LNMLFTGLFTVEMILKLIAFKP PWNVFDFLIVIGSIIDVILS ITFFRLFRVMRLVKLLSRGEGIRT
α1D SPFEYMMFVLIMLNTLCLAMQHY LNMVFTGVFTVEMVLKVIAFKP AWNTFDSLIVIGSIIDVALS ITFFRLFRVMRLVKLLSRGEGIRT
α1A PPFEYTIMAMIALNTIVLMMKFY FNIVFTSLFSLECLLKVLAFGI AWNIFDFVTVLGSITDILVT LSFLRLFRAARLIKLLRQGYTIRI
α1B PPFEYFIMAMIALNTVVLMMKFY LNIVFTSMFSMECVLKIIAFGV AWNVFDFVTVLGSITDILVT LSFLRLFRAARLIKLLRQGYTIRI
α1E PSFEYTIMAMIALNTVVLMMKYY LNIAFTMVFSLECVLKVIAFGF TWNIFDFITVIGSITEIVLT MSFLKLFRAARLIKLLRQGYTIRI
Na Nabr1 QVFDISIMILICLNMVTMMVETD INLVFIVLFTGECVLKLISLRH GWNIFDFVVVILSIVGMFLA FRVIRLARIGRILRLIKGAKGIRT
Nabr2 QVFDISIMILICLNMVTMMVETD INLVFIVLFTGECVLKLISLRH GWNIFDFVVVILSIVGMFLA FRVIRLARIGRILRLIKGAKGIRT
Nabr3 QVFDISIMILICLNMVTMMVETD INLVFIVLFTGEFLLKLISLRY GWNIFDFVVVILSIVGMFLA FRVIRLARIGRILRLIKGAKGIRT
Nabr6 QAFDIVIMMLICLNMVTMMVETD INLVFVIFFTCECVLKMFALRH GWNIFDFVVVILSIVGMFLA FRVIRLARIGRILRLIKGAKGIRT
Naskm QVFDISIMILICLNMVTMMVETD INMVFIIIFTGECVLKMFALRH GWNIFDFVVVILSIVGLALS FRVIRLARIGRVLRLIRGAKGIRT
Naht QAFDVTIMFLICLNMVTMMVETD INLLFVAIFTGECIVKMAALRH SWNIFDFVVVILSIVGTVLS FRVIRLARIGRILRLIRGAKGIRT
NaSNS QAFDIIIMVLICLNMITMMVETD INQFFVAVFTGECVMKMFALRQ GWNVFDFIVVILSIGSLLFS FRVIRLARIGRILRLIRAAKGIRT
Na2.1 QAFNVIVMVLICFQAIAMMIDTD INSIFVMLYTMECILKLIAFRC AWNIFDFMVVIFSITGLCLP VQLILLSRIIHMLRLGKGPKVFHN


S5
P Loop
S6
Domain I
. n n cc@.nc c c @ n #
@ c. . .cc. n@c@ n c
c@c.c#n. . @ . n@ c
Rb21 QIWSVSIFLLFFLLLYGILGVQMFGTF FNEIGTSIFTVYEASSQEGWVFLMY WRSYFYFITLIFFLAWLVKNVFIAVIIETF
ceC27 QIQNVTIFFMFFVFSYAIMGVQLFGRL FSDFASSLFTVYLAASQEGWVYVLY FLAFFYFVTLIFFLAWLVKNVFIAVITETF
Ca α1S PLFHIALLVLFMVIIYAIIGLELFKGK FDNFGFSMLTVYQCITMEGWTDVLY EWPWIYFVTLILLGSFFILNLVLGVLSGEF
α1C PLLHIALLVLFVIIIYAIIGLELFMGK FDNFAFAMLTVFQCITMEGWTDVLY ELPWVYFVSLVIFGSFFVLNLVLGVLSGEF
α1D PLLHIALLVLFVIIIYAIIGLELFIGK FDNFAFAMLTVFQCITMEGWTDVLY ELPWVYFVSLVIFGSFFVLNLVLGVLSGEF
α1A PLLQIGLLLFFAILIFAIIGLEFYMGK FDNILFAVLTVFQCITMEGWTDLLY TWNWLYFIPLIIIGSFFMLNLVLGVLSGEF
α1B PLLQIGLLLFFAILMFAIIGLEFYMGK FDNILFAILTVFQCITMEGWTDILY TWNWLYFIPLIIIGSFFMLNLVLGVLSGEF
α1E PLLQIGLLLFFAILMFAIIGLEFYSGK FDNILFAVLTVFQCITMEGWTTVLY TWNWLYFIPLIIIGSFFVLNLVLGVLSGEF
Na Nabr1 KLSDVMILTVFCLSVFALIGLQLFMGN FDTFSWAFLSLFRLMTQDFWENLYQ KTYMIFFVLVIFLGSFYLINLILAVVAMAY
Nabr2 KLSDVMILTVFCLSVFALIGLQLFMGN FDTFSWAFLSLFRLMTQDFWENLYQ KTYMIFFVLVIFLGSFYLINLILAVVAMAY
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Nabr6 KLSDVMILTVFCLSVFALIGLQLFHGN FDTFSWAFLALFRLMTQDYWENLYQ KTYMIFFVLVIFVGSFYPVNLILAVVAMAY
Naskm KLSDVMILTVFCLSVFALVGLQLFMGN YDTFSWAFLALFRLMTQDYWENLFQ KTYMIFFVVIIFLGSFYLINLILAVVAMAY
Naht KLADVMVLTVFCLSVFALIGLQLFMGN FDSFAWAFLALFRLMTQDCWERLYQ KIYMIFFMLVIFLGSFYLVNLILAVVAMAY
NaSNS KLADVTILTVFCLSVFALVGLQLFKGN FDSFAWAFLSLFRLMTQDSWERLYQ KMYMVFFVLVIFLGSFYLVNLILAVVTMAY
Na2.1 QLIGVIILTLFFLSIFSLIGMGLFMGN FDSFGWALFALFRLMAQDYPEVLYH KVYMIFFVVVSFLFSFYMASLFLGILAMAY
Domain II
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c @cncn . @cc@c @ c .ccn
.c.cc@c. cc ncc@ n@ @ nccc
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ceC27 KLGGLVIFTGILLIVTSAISLQLFCYV FTNFAVAFMSMFQIITQEGWTDVVI PFVAIYFVAYHLLVTLFVLSLFVAVILINL
Ca α1S SIASLLLLLFLFIIIFALLGMQLFGGR FDNFPQALISVFQVLTGEDWNSVMY VLVCIYFIILFVCGNYILLNVFLAIAVINL
α1C SIASLLLLLFLFIIIFSLLGMQLFGGK FDNFPQSLLTVFQILTGEDWNSVMY MLVCIYFIILFICGNYILLNVFLAIAVINL
α1D SSASLLLLLFLFIIIFSLLGMQLFGGK FDNFPQALLTVFQILTGEDWNAVMY MIVCIYFIILFICGNYILLNVFLAIAVINL
α1A SIISLLFLLFLFIVVFALLGMQLFGGQ FDTFPAAIMTVFQILTGEDWNEVMY MVFSIYFIVLTLFGNYTLLNVFLAIAVINL
α1B SIISLLFLLFLFIVVFALLGMQLFGGQ FDTFPAAILTVFQILTGEDWNAVMY MFSSFYFIVLTLFGNYTLLNVFLAIAVINL
α1E SIISLLFLLFLFIVVFALLGMQLFGGR FDTFPAAIMTVFQILTGEDWNEVMY MWSAVYFIVLTLFGNYTLLNVFLAIAVINL
Na Nabr1 ALGNLTLVLAIIVFIFAVVGMQLFGKS MNDFFHSFLIVFRVLCGEWIETMWD AMCLTVFMMVMVIRNLVVLNLFLALLLSSF
Nabr2 ALGNLTLVLAIIVFIFAVVGMQLFGKS MHHFFHSFLIVFRVLCGEWIETMWD TMCLTVFMMVMVIGNLVVLNLFLALLLSSF
Nabr3 ALGNLTLVLAIIVFIFAVVGMQLFGKS MNDFFHSFLIVFRVLCGEWIETMWD TMCLIVFMLVMVIGNLVVLNLFLALLLSSF
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Naht ALGNLTLVLAIIVFIFAVVGMQLFGKN MMDFFHAFLIIFRILCGEWIETMWD SLCLLVFLLVMVIGNLVVLNLFLALLLSSF
NaSNS ALGNLTFILAIIVFIFALVGKQLLSED MCDFFHSFLVVFRILCGEWIENMWV SICLILFLTVMVLGNLVVLNLFIALLLNSF
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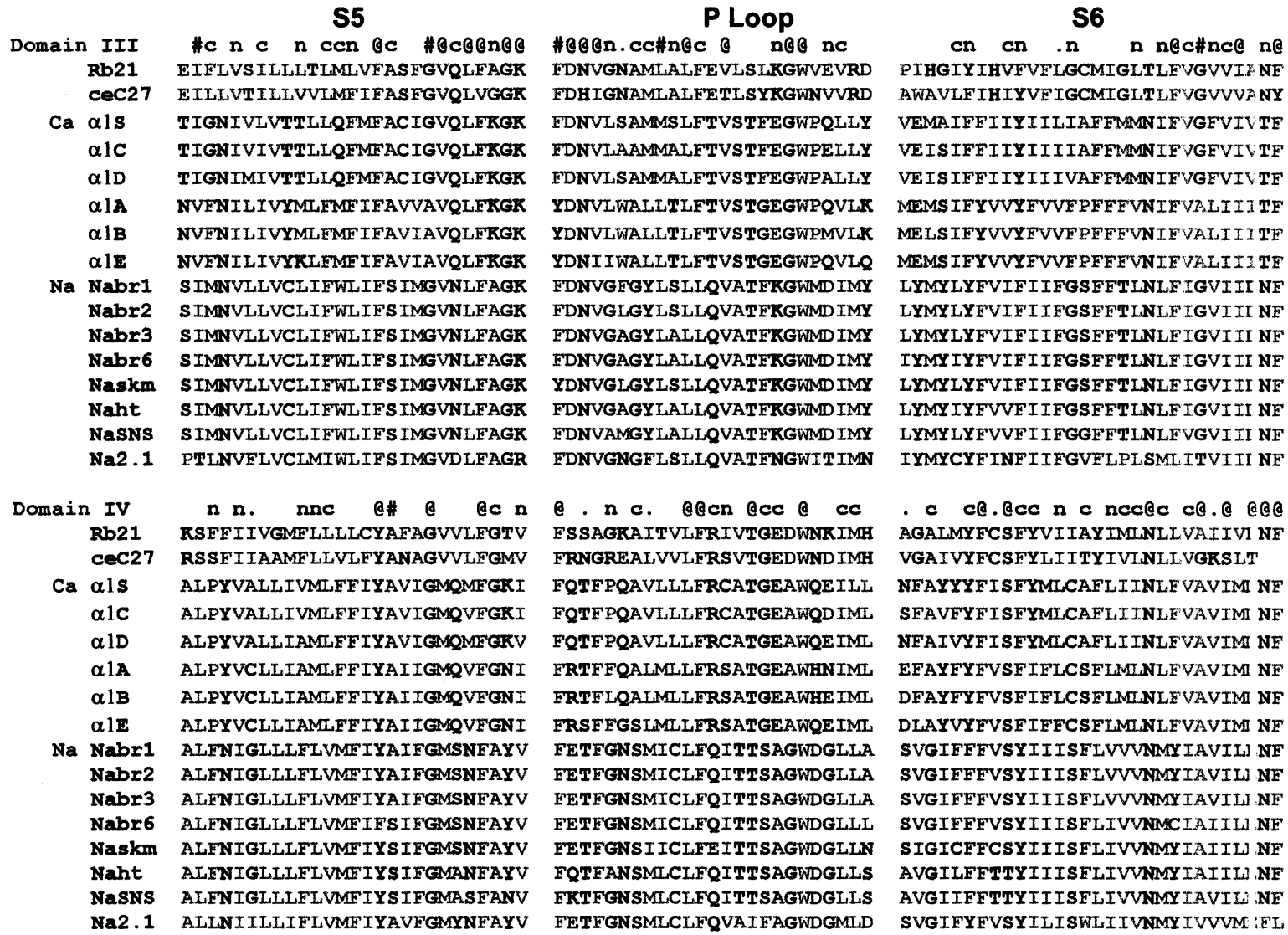
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S6
Domain III
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ceC27 EILLVTILLVVLMFIFASFGVQLVGGK FDHIGNAMLALFETLSYKGWNVVRD AWAVLFIHIYVFIGCMIGLTLFVGVVVF NY
Ca α1S TIGNIVLVTTLLQFMFACIGVQLFKGK FDNVLSAMMSLFTVSTFEGWPQLLY VEMAIFFIIYIILIAFFMMNIFVGFVIV TF
α1C TIGNIVIVTTLLQFMFACIGVQLFKGK FDNVLAAMMALFTVSTFEGWPELLY VEISIFFIIYIIIIAFFMMNIFVGFVIV TF
α1D TIGNIMIVTTLLQFMFACIGVQLFKGK FDNVLSAMMALFTVSTFEGWPALLY VEISIFFIIYIIIVAFFMMNIFVGFVIV TF
α1A NVFNILIVYMLFMFIFAVVAVQLFKGK YDNVLWALLTLFTVSTGEGWPQVLK MEMSIFYVVYFVVFPFFFVNIFVALIII TF
α1B NVFNILIVYMLFMFIFAVIAVQLFKGK YDNVLWALLTLFTVSTGEGWPMVLK MELSIFYVVYFVVFPFFFVNIFVALIII TF
α1E NVFNILIVYKLFMFIFAVIAVQLFKGK YDNIIWALLTLFTVSTGEGWPQVLQ MEMSIFYVVYFVVFPFFFVNIFVALIII TF
Na Nabr1 SIMNVLLVCLIFWLIFSIMGVNLFAGK FDNVGFGYLSLLQVATFKGWMDIMY LYMYLYFVIFIIFGSFFTLNLFIGVIII NF
Nabr2 SIMNVLLVCLIFWLIFSIMGVNLFAGK FDNVGLGYLSLLQVATFKGWMDIMY LYMYLYFVIFIIFGSFFTLNLFIGVIII NF
Nabr3 SIMNVLLVCLIFWLIFSIMGVNLFAGK FDNVGAGYLALLQVATFKGWMDIMY LYMYLYFVIFIIFGSFFTLNLFIGVIII NF
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Naskm SIMNVLLVCLIFWLIFSIMGVNLFAGK YDNVGLGYLSLLQVATFKGWMDIMY LYMYLYFVIFIIFGSFFTLNLFIGVIII NF
Naht SIMNVLLVCLIFWLIFSIMGVNLFAGK FDNVGAGYLALLQVATFKGWMDIMY LYMYIYFVVFIIFGSFFTLNLFIGVIII NF
NaSNS SIMNVLLVCLIFWLIFSIMGVNLFAGK FDNVAMGYLALLQVATFKGWMDIMY LYMYLYFVVFIIFGGFFTLNLFVGVIII NF
Na2.1 PTLNVFLVCLMIWLIFSIMGVDLFAGR FDNVGNGFLSLLQVATFNGWITIMN IYMYCYFINFIIFGVFLPLSMLITVIII NF
Domain IV
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Rb21 KSFFIIVGMFLLLLCYAFAGVVLFGTV FSSAGKAITVLFRIVTGEDWNKIMH AGALMYFCSFYVIIAYIMLNLLVAIIVI NF
ceC27 RSSFIIAAMFLLVLFYANAGVVLFGMV FRNGREALVVLFRSVTGEDWNDIMH VGAIVYFCSFYLIITYIVLNLLVGKSLT
Ca α1S ALPYVALLIVMLFFIYAVIGMQMFGKI FQTFPQAVLLLFRCATGEAWQEILL NFAYYYFISFYMLCAFLIINLFVAVIMI NF
α1C ALPYVALLIVMLFFIYAVIGMQVFGKI FQTFPQAVLLLFRCATGEAWQDIML SFAVFYFISFYMLCAFLIINLFVAVIMI NF
α1D ALPYVALLIAMLFFIYAVIGMQMFGKV FQTFPQAVLLLFRCATGEAWQEIML NFAIVYFISFYMLCAFLIINLFVAVIMI NF
α1A ALPYVCLLIAMLFFIYAIIGMQVFGNI FRTFFQALMLLFRSATGEAWHNIML EFAYFYFVSFIFLCSFLMLNLFVAVIMI NF
α1B ALPYVCLLIAMLFFIYAIIGMQVFGNI FRTFLQALMLLFRSATGEAWHEIML DFAYFYFVSFIFLCSFLMLNLFVAVIMI NF
α1E ALPYVCLLIAMLFFIYAIIGMQVFGNI FRSFFGSLMLLFRSATGEAWQEIML DLAYVYFVSFIFFCSFLMLNLFVAVIMI NF
Na Nabr1 ALFNIGLLLFLVMFIYAIFGMSNFAYV FETFGNSMICLFQITTSAGWDGLLA SVGIFFFVSYIIISFLVVVNMYIAVILI NF
Nabr2 ALFNIGLLLFLVMFIYAIFGMSNFAYV FETFGNSMICLFQITTSAGWDGLLA SVGIFFFVSYIIISFLVVVNMYIAVILI NF
Nabr3 ALFNIGLLLFLVMFIYAIFGMSNFAYV FETFGNSMICLFQITTSAGWDGLLA SVGIFFFVSYIIISFLVVVNMYIAVILI NF
Nabr6 ALFNIGLLLFLVMFIFSIFGMSNFAYV FETFGNSMICLFQITTSAGWDGLLL SVGIFFFVSYIIISFLIVVNMCIAIILI NF
Naskm ALFNIGLLLFLVMFIYSIFGMSNFAYV FETFGNSIICLFEITTSAGWDGLLN SIGICFFCSYIIISFLIVVNMYIAIILI NF
Naht ALFNIGLLLFLVMFIYSIFGMANFAYV FQTFANSMLCLFQITTSAGWDGLLS AVGILFFTTYIIISFLIVVNMYIAIILI NF
NaSNS ALFNIGLLLFLVMFIYSIFGMASFANV FKTFGNSMLCLFQITTSAGWDGLLS AVGIIFFTTYIIISFLIVVNMYIAVILI NF
Na2.1 ALLNIILLIFLVMFIYAVFGMYNFAYV FETFGNSMLCLFQVAIFAGWDGMLD SVGIFYFVSYILISWLIIVNMYIVVVM FL

Figure 14. Alignment of the Rb21-channel, a putative *C. elegans* channel (CEL27f2), and reported Ca^{2+} and Na^{+} channels. The putative transmembrane segments of the Rb21-channel and the *C. elegans* channel were obtained from hydropathicity analyses and sequence comparison with calcium and sodium channels of which putative membrane spanning portions were reported previously (Jan and Jan, 1990; Perez-Reyes and Schneider, 1994). Each potential transmembrane sequence was selected to have a equal size. Amino acid residues are colored according to their following properties: positively-charged residues are red; negatively-charged residues are green; hydrophobic residues are yellow; and hydrophilic residues are blue. Conserved amino acids found in the Rb21-channel and reported calcium and sodium channel are marked as follows: amino acid residues in the Rb21-channel identical to at least 13 channels out of 14 channels are marked as "@"; amino acid residues in the Rb21-channel identical to less than 13, but at least 3 out of 6 calcium channels and 4 out of 8 sodium channels are marked as "#"; amino acid residues in the Rb21-channel found in at least 3 out of 6 calcium channels, but less than 4 out of 8 sodium channels are marked as "c"; amino acid residues in the Rb21-channel found in at least 4 out of 8 sodium channels, but less than 3 out of 6 calcium channels are marked as "n"; identical amino acids in the Rb21-channel found in at least 1 channels, but less than 3 channels out of 12 calcium and sodium channels are marked as ".". Sources of published calcium and sodium channels are as follows: α1S is from rabbit skeletal muscle, M23919 (Tanabe *et al.*, 1987); α1C is from human heart, L04569 (Schultz *et al.*, 1993); α1D is from human neuroblastoma cell line, M76558 (Williams *et al.*, 1992b); α1A is from rat brain, (Mori *et al.*, 1991); α1B is from human brain, M94172 (Williams *et al.*, 1992a); α1E is from human brain, L27745 (Schneider *et al.*, 1994); Nabr1 is from rat brain, X03638 (Noda *et al*, 1986); Nabr2 is from rat brain, M22254 (Noda and Numa,1987); Nabr3 is from rat brain, Y00766 (Numa *et al*, 1988); Nabr6 is from rat brain, L39018 (Schaller *et al.*, 1995); Naskm is from rat skeletal muscle, M26643 (Trimmer *et al.*, 1989); Naht is from rat heart, M29709 (Rogart *et al.*, 1989); NaSNS is from rat sensory neurons, G1209466 (Wood et al., 1996); and Na2.1 is from human brain, M91556 (George *et al.*, 1992).

of a Ca^{2+} channel into a positively charged amino acid changed a Ca^{2+}-selective channel into a monovalent cation-selective channel which is not permeable to divalent ions (Parent and Gopalakrishnan, 1995).

Compared with Ca^{2+} or Na^{+} channels, pore loops of the Rb21-channel contains very conserved amino acids found in both channels. The Rb21-channel contains EEKE residues (E, glutamate; K, lysine) in the pore loops of 4 domains, which are similar to selectivity determining residues of Ca^{2+} or Na^{+} channels. The negatively-charged residues (E) of domain I and IV are identical to those of Ca^{2+} channels (EEEE), while the positively-charged K of domain III is identical that of Na^{+} channels (DEKA). These results suggested that the Rb21-channel may have similar properties to either a Ca^{2+} or a Na^{+} channel in terms of ion selectivity. Or the putative channel might be a distinctive cation channel, which is nonselectively permeable to most cation ions, such as Na^{+}, Ca^{2+}, Mg^{2+}, and K^{+}.

4.5 Phylogenetic relationship of the Rb21-channel with Ca^{2+} and Na^{+} channels

The conclusion that the Rb21-channel may be a cation-selective ion channel was drawn from hydropathy analysis, matrix homology plots, and sequence alignment between the Rb21 channel, and Ca^{2+} and Na^{+} channels. Evolutionary relationships between Rb21 channel, Ca^{2+} and Na^{+} channels were analyzed using Higgins and Sharp algorithm (1988).

Amino acid sequences of only putative membrane spanning regions (figure 14) were compared to predict evolutionary relationships on the basis of sequence similarity between the Rb21-channel, and Ca^{2+} and Na^{+} channels. The algorithm constructed sequence alignments progressively from most homologous sequences to lesser ones. Alignment of two

sequences produced a consensus file, which was then compared to other sequences or consensus files.

The phylogenetic analysis (Higgins and Sharp, 1988) grouped the aligned sequences into the three families, Ca^{2+} channel, Na^{+} channel, and Rb21-C27 channel families (figure 15). In the relationships between the three families, the Ca^{2+} channel family was more closely grouped with the Na^{+} channel family than the Rb21-C27 family. Phylogenetic relationships suggested that Rb21-channel might have diverged earlier than both Ca^{2+} and Na^{+} channels from an ancestral 4-domain channel, or that Rb21-channel might be an ancestor of Ca^{2+} and Na^{+} channels.

4.6 Distribution of Rb21-channel mRNA

Northern blot analyses were performed to localize the expression of the Rb21-channel mRNA. A multiple tissue blot was prepared from total RNAs isolated from rat heart, brain, skeletal muscle, liver, lung, kidney, and testis (figure 16A). Human multiple tissue blots were purchased from Clontech (figure 16B and C). The probe was synthesized from the cDNA containing the domain III, IV, and 3'-tail of the Rb21 cDNA. Specificity of the probe was checked by searching for homologous sequences to the probe cDNA in the Genbank database using the BLAST program. No similar sequences were detected.

Results of Northern blot analyses showed that the size of detected transcripts was about 6.9 kilobases. This size was similar to the cDNA construct of the Rb21-channel, 6820 bases, suggesting that the full-length cDNA was cloned. Transcripts of the Rb21-channel were detected in brain, heart,and pancreas (figure 16A and B). On the contrary, transcripts

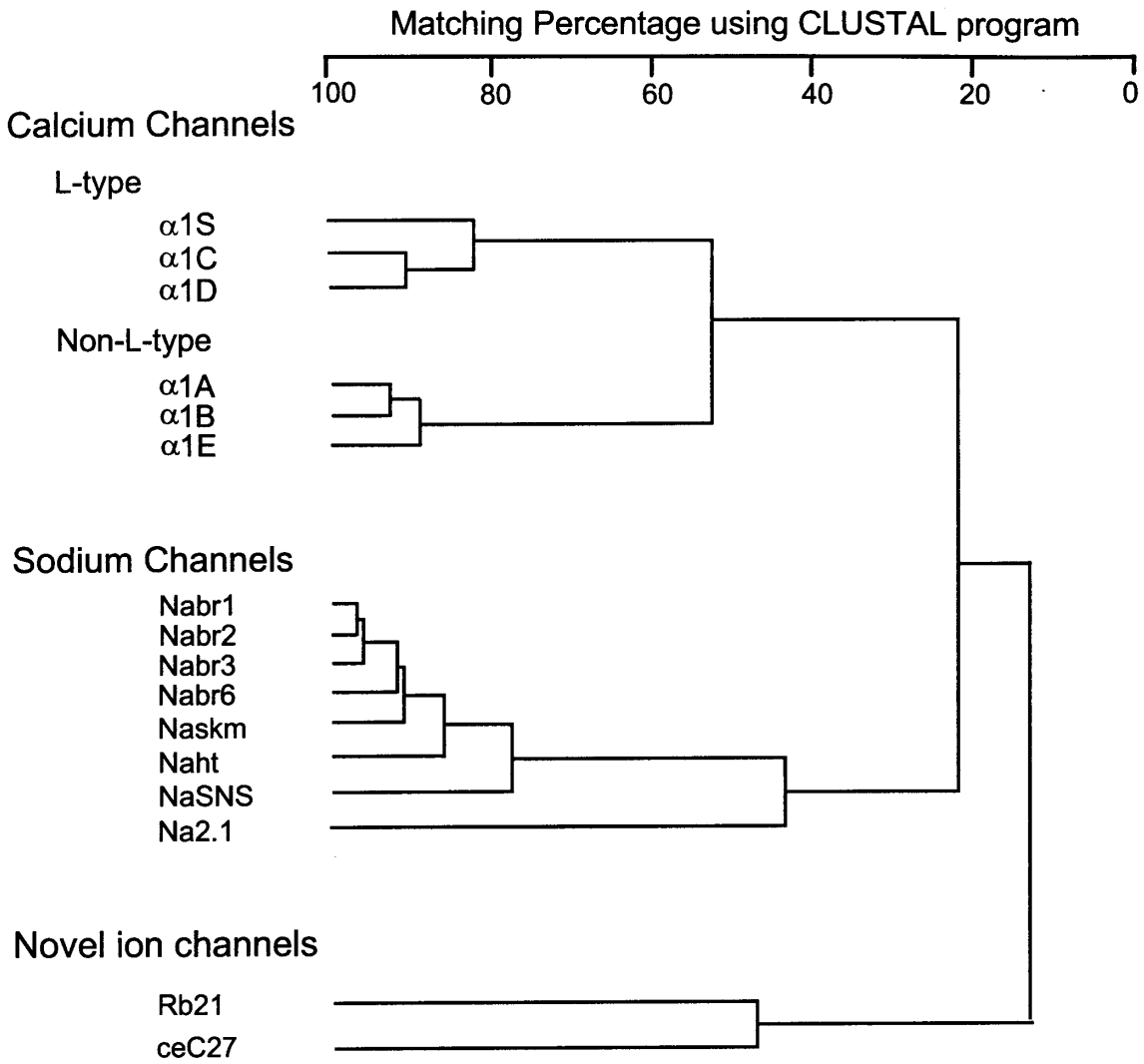


Figure 15. A phylogenetic tree of the Rb21-channel with Ca^{2+} and Na^{+} channels. To predict evolutionary relationships between the Rb21-channel, and calcium and sodium channels, amino acid sequences of only putative membrane spanning regions in figure 14 were compared to one another using the Higgins and Sharp algorithm (1988). Sources of calcium and sodium channels are given in the legend of figure 14.

were not detected in lung, skeletal muscle, testis, kidney, liver, and placenta. In brain regions, the Rb21-channel was expressed in the following order: amygdala = corpus callosum > caudate nucleus > hippocampus > substantia nigra = subthalamic nucleus > thalamus.

A single band of about 6.9 kilobases was detected from the rat multiple tissue blot (figure 16A), while two smaller bands (about 4.5 and 1.5 kilobases) were detected in the human multiple tissue blot (figure 16B). Possible interpretations were that the smaller bands could be alternate splicing variants of Rb21 channel transcripts, or nonspecific signals such as ribosomal RNAs. Smaller bands were not detected in the rat multiple tissue blot for which probes were washed at a high stringency condition (68°C at a 0.1 X SSC and 0.1 % SDS solution), but in human blots for which the probes were washed in a moderate stringent condition (60°C at a 0.5 X SSC and 0.1 % SDS solution). Sizes of the small bands (4.5 and 1.5 kilobases) were similar to those of ribosomal RNA, suggesting that small bands might be nonspecific signals such as ribosomal RNAs.

4.7 Expression studies of the Rb21-channel

4.7a Characterization of native *Xenopus* oocyte Ca^{2+} channel currents

Figure 17A shows representative traces of endogenous Ca^{2+} channel currents measured from 0.1 M KCl injected oocytes. Peak currents of native Ca^{2+} channels ranged from 0 to 20 nA in 40 Ba^{2+} solution (n=24 from 12 batches). The injection of Ca^{2+} channel ancillary subunits, either β2, β4, β2 plus α2, or β4 plus α2, stimulated endogenous oocyte currents (n=86, 13 out of 15 batches). Peak amplitudes of the stimulated currents varied

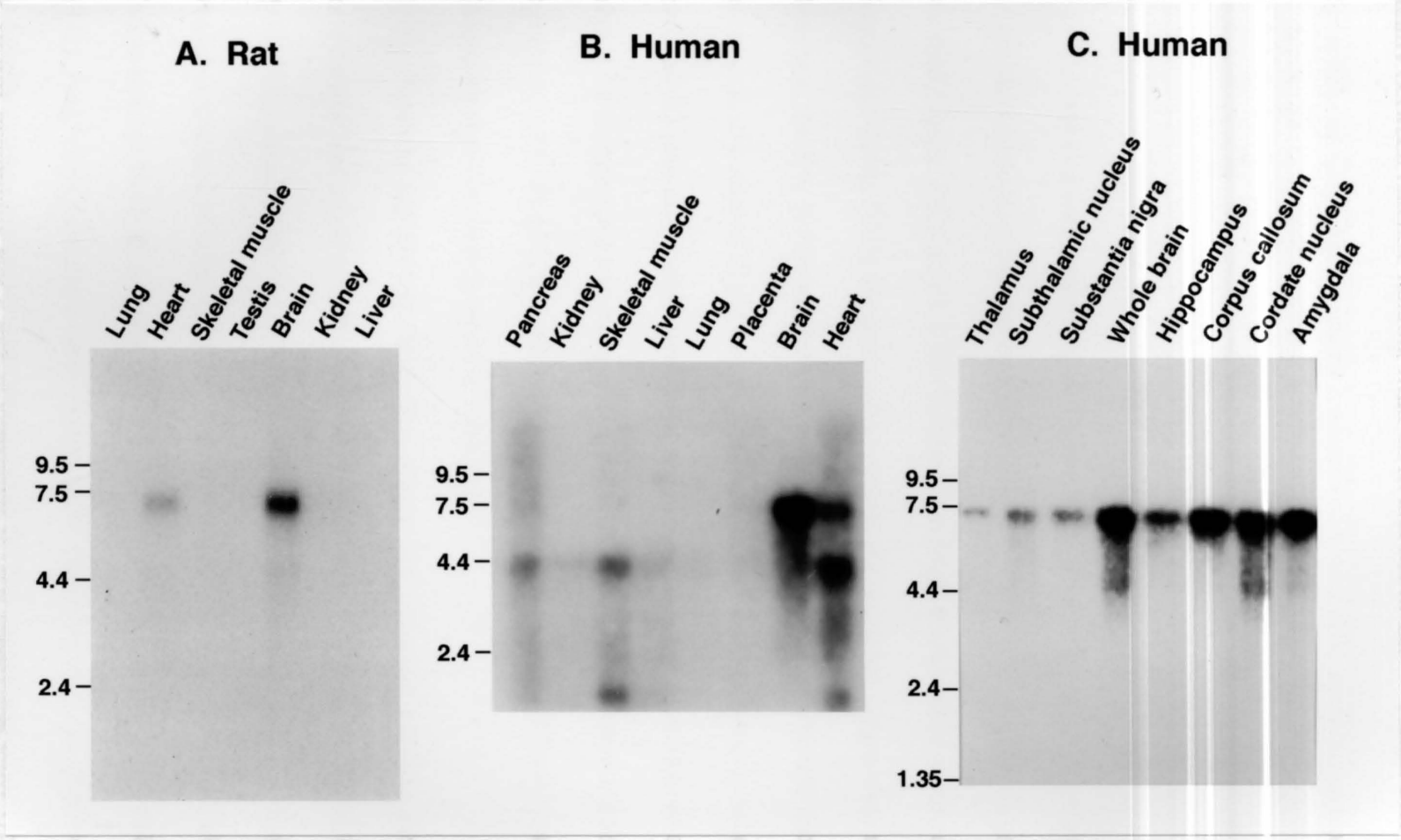


Figure 16. Northern blot analyses of Rb21-channel mRNA in rat (A) and human multiple tissues (B and C). In A, 20 μg of total RNA isolated from rat tissues were separated on a denaturation gel. The separated RNA were transferred, immobilized, and hybridized with ^{32}P-labeled probes. Human tissue blots were purchased from Clontech (B and C). Each lane contains 2 μg of poly(A)$^+$ RNA. The probe was synthesized from a cDNA containing domain III, IV, and C-terminal tail of the Rb21-channel.

greatly between batches, ranging from 40 to 200 nA of Ba^{2+}. Figure 17B shows a set of representative Ca^{2+} channel currents stimulated by β2 plus α2. In that batch, auxiliary subunits increased native oocyte currents by about 10-fold. From the other 2 (n=6) out of 15 batches, however, oocytes did not express Ca^{2+} channel currents even after injection of ancillary subunits.

Amplified oocyte currents were dissected pharmacologically. Bay K 8644 (μM), an L-type Ca^{2+} channel agonist, increased β2 plus α2-amplified Ca^{2+} channel currents by about two-fold (figure 17C), and shifted the current-voltage relationships to negative potentials (Rampe and Kane, 1994), suggesting the presence of functional L-type channels in oocytes. However, the Bay K 8644-stimulated currents could only be measured from 3 out of 8 batches. Application of 10 μM nisoldipine blocked 10.6 ± 5.3 % (mean ± standard deviation, n=7) of β2 plus α2-amplified currents, suggesting that the percentage of control current carried by L-type channels were about 10 %. These results were consistent with the expression of α1S, α1C, and α1D mRNA as detected by PCR (figure 2), suggesting that *Xenopus* oocytes express L-type Ca^{2+} channels.

Previous reports described that N-type Ca^{2+} channel currents can be a major component of endogenous oocyte currents (Lacerda *et al.*, 1993; Perez-Reyes and Schneider, 1994). The presence of N-type channel currents in β2- plus α2-stimulated oocyte currents were characterized with the components blocked by 3 μM ω-conotoxin GVIA (figure 17D). The percentage block by ω-conotoxin GVIA ranged from 21 to 64 % (32.8 % ± 13.2, mean ± S. D., n=15 from 4 batches). Significant residual currents were not blocked by a mixture of 10 μM nisoldipine and 3 μM ω-conotoxin GVIA, suggesting other types of calcium

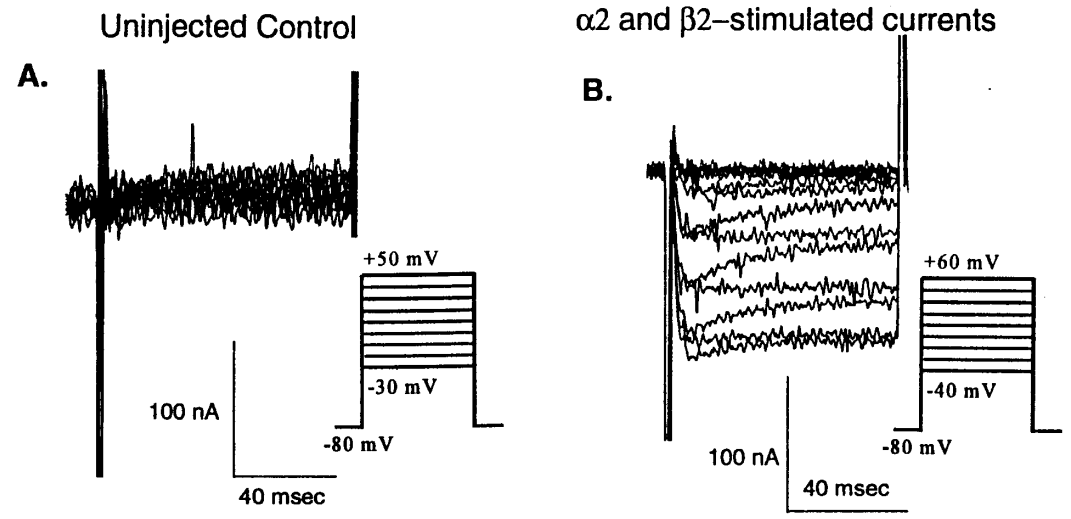
channels such as R-type might be expressed in oocytes.

Taken together, endogenous L-type and N-type currents were detected in oocytes by standard pharmacological criteria from oocyte native Ca^{2+} channel currents. N-type currents were predominantly expressed, while L-type currents were rarely detected in oocytes. In keeping with PCR results showing the expression of α1A and α1E mRNA, I conclude that the currents resistant to 10 μM nisoldipine and 3 μM ω-conotoxin GVIA might be P/Q- and R-type currents.

4.7b Expression of the Rb21-channel in *Xenopus* oocytes

As positive controls for expression of cloned VACCs, rabbit α1C and human α1E clones were expressed in oocytes. For the expression of rabbit cardiac L-type channels, rabbit α1C-cRNA was coinjected with α2 and β2 cRNA. Significant Ba^{2+} currents were not detected from uninjected control oocytes (n=27 from 5 batches) in the 40mM Ba^{2+} solution. On the contrary, robust L-type currents (3.62 μA ± 0.88, mean ± S. D., n=32 from 5 batches) were measured from the oocytes injected with α1C-, α2-, and β2-cRNA (figure 18A). Also, R-type currents (2.79μA ± 0.72, mean ± S. D., n=13 from 5 batches) were measured from the oocytes injected with human α1E-cRNA (figure 18B).

Expression of the Rb21-channel has been attempted in *Xenopus* oocytes. Rb21-channel cRNA synthesized *in vitro* was injected into *Xenopus* oocytes. Currents were measured from the 3rd day to the 14th day after injection. Because the Rb21-channel shares similarity with both Ca^{2+} and Na^{+} channels in structure, the putative channel may be permeable to either or both cation. Therefore, currents were measured in two external


Uninjected Control
A.
+50 mV
-30 mV
-80 mV
100 nA
40 msec
α2 and β2–stimulated currents
B.
+60 mV
-40 mV
-80 mV
100 nA
40 msec

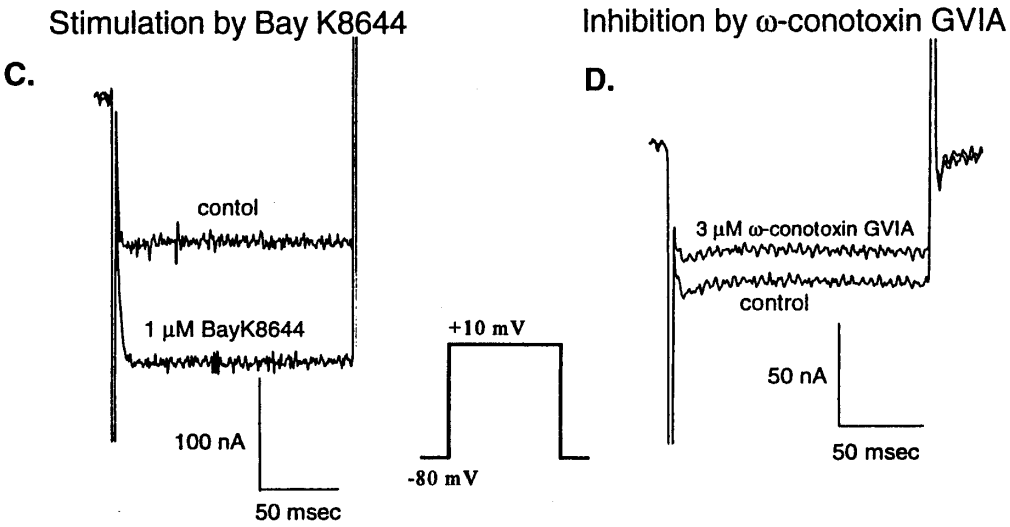

Stimulation by Bay K8644
C.
contol
1 μM BayK8644
+10 mV
-80 mV
100 nA
50 msec
Inhibition by ω-conotoxin GVIA
D.
3 μM ω-conotoxin GVIA
control
50 nA
50 msec

Figure 17. Electrophysiological and pharmacological characterization of native oocyte calcium channel currents. In (A), endogenous oocyte calcium channel currents were measured from oocytes injected with 0.1 M KCl solution. Test pulses of -30 to +50 mV were applied from a holding potential of -80 mV. In (B), endogenous oocyte calcium channel currents were measured from oocytes injected with cRNAs of calcium channel auxiliary subunits, $\alpha 2$ and $\beta 2$. Currents were measured from the 5th day after injection of the cRNAs in 40 mM Ba^{2+} solution. Test pulses were -40 to +60 mV from a holding potential of -80 mV. Effects of 1 μM Bay K8644 (C) and 3 μM ω-conotoxin GVIA (D) were examined on $\alpha 2$- plus $\beta 2$-stimulated oocyte currents. Test pulses of 20 mV were applied from a holding potential of -80 mV every 20 sec.

solutions: SOS containing 100 mM Na^+ for Na^+ channel currents, or 40 mM Ba^{2+} solution for Ca^{2+} channel currents. However, currents measured from Rb21 cRNA-injected oocytes were indistinguishable from those of uninjected oocytes (figure 18C and D), suggesting that Rb21-channels did not express as voltage-activated Ca^{2+} or Na^+ channels. These results are summarized in table 7.

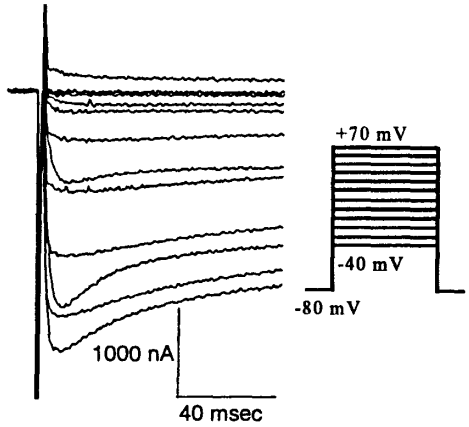
	Negative control	**Positive control** (α1C and α1E)	**Rb21-channel**
Number of samples	27 cells out of 5 batches	45 cells out of 5 batches	82 cells out of 5 batches
Expression	No significant current	Robust currents	No significant current

Table 7. Summary of Rb21-channel expression experiments in *Xenopus* oocytes. For the negative control, currents were measured from uninjected or 0.1 M KCl-injected oocytes in both SOS and 40 mM Ba^{2+} solution. For the positive control, currents were measured from α1C- and α1E-injected oocytes in either 40 mM Ba^{2+} solution or 2 mM Ba^{2+} solution. The expression of the Rb21-channel was attempted in either SOS, 40 mM Ba^{2+} solution, or 100 Li^+ solution.

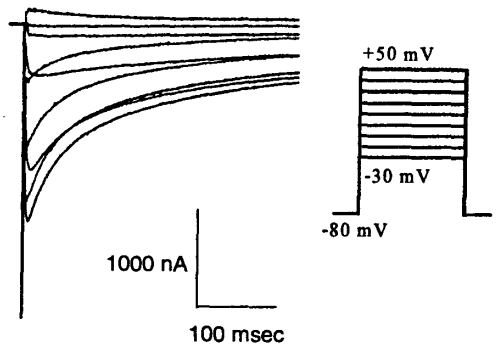
Hyperpolarization-activated calcium channel currents were characterized from *Paramecium* (Preston *et al.*, 1992). Hyperpolarization-activated cation currents (I_f and I_h) were identified from heart and brain (DiFrancesco, 1993; Pape, 1996). To test the possibility that Rb21-channel is a hyperpolarization-activated channel, currents were measured with a protocol for which test potentials of -50 to -130 mV were applied from a holding potential of -40 mV in both SOS and 40 mM Ba^{2+} solution (n=10). In 40 mM Ba^{2+} solution, no detectable currents from both Rb21-injected and uninjected oocytes were evoked by the voltage protocol.

Another hypothesis tested was that Rb21-channel is a voltage-activated cation

A. $\alpha 1C\alpha 2\beta 2$
in 40 Ba solution

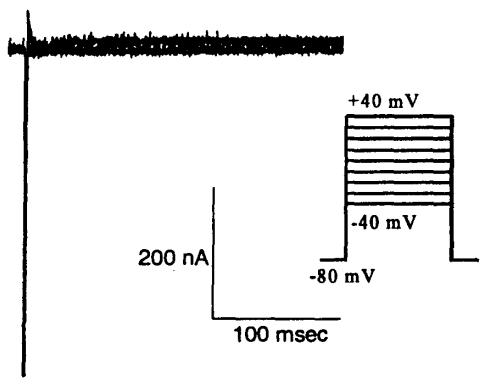


B. $\alpha 1E$ alone
in 2 Ba solution



C.

Rb21-channel
in SOS



D.

Rb21-channel
in 40 Ba solution

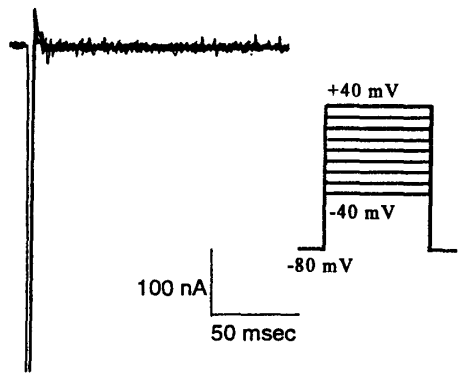


Figure 18. Electrophysiological measurements to detect the expression of the Rb21-channel in *Xenopus* oocytes by the two-microelectrode voltage clamp method. Currents were measured from the fourth day after injection of cRNAs. As a positive control, the rabbit cardiac α1C was expressed with auxiliary subunits, α2 and β2 (A). Currents were evoked by a series of test pulses of -40 to +60 mV from a holding potential of -80 mV in 40 mM Ba^{2+} solution. The expression of the human α1E was measured in (B). Currents were evoked by test pulses of -30 to +50 mV from a holding potential of -90 mV in 2 mM Ba^{2+} solution (B). The expression of Rb21-cRNA were tested in SOS solution (C) and in 40 mM Ba^{2+} solution (D). Test pulses were -40 to +40 mV from a holding potential of -80 mV.

channel which is permeable to monovalent cations. Because divalent ions such as Ca^{2+} and Mg^{2+} could block the possible cation channels, currents were also measured in 100 Li^{+} solution (100 mM LiCl, 25 mM TEA-Cl, and 10 mM HEPES, pH=7.4 adjusted with TEA-OH). However, currents measured from oocytes injected with Rb21-channel cRNA were not distinguishable from those of uninjected oocytes. Taken together, expression of the Rb21-channel was not detected as a voltage-activated current in *Xenopus* oocytes.

4.7c Expression of the Rb21-channel in HEK 293 cells

Initial studies of the rabbit skeletal muscle Ca^{2+} channel $\alpha 1$ failed to detect expression in *Xenopus* oocytes. But the channel was expressed in mouse L-cells and dysgenic myotubules (Perez-Reyes *et al.*, 1989; Tanabe *et al.*, 1988). Although the human $\alpha 1D$ was first reported to be expressed in *Xenopus* oocytes, the expressed currents were poor (Williams *et al.*, 1992). Recently, preliminary data suggests that a human $\alpha 1D$ was convincingly expressed in HEK 293 cells (Hans *et al.*, 1997). By analogy, the Rb21-channel might be expressed in other systems, such as tsA201 cells.

The cDNA of the Rb21-channel was subcloned into a mammalian expression vector, pTracer (Invitrogen). This plasmid construct was transfected into tsA201 cells using Lipofectamine reagent (Promega). Transfected cells were detected by expression of green fluorescence, which is produced by a jellyfish **g**reen **f**luorescence **p**rotein (GFP) gene in the vector.

As a negative control, pTracer vectors not containing any inserts were transfected into tsA201 cells. No significant currents were measured from transfected cells in either Tyrode

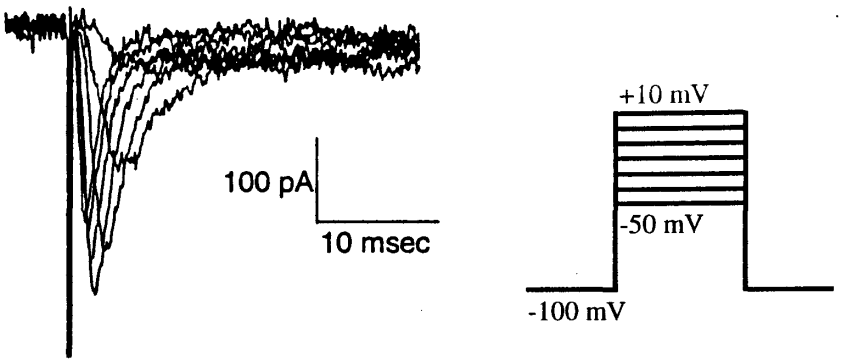
or 10 mM Ba^{2+} solutions (n=17 out of 4 batches). Tyrode solution containing 140 mM Na^+ was used to measure Na^+ channel currents, while 10 mM Ba^{2+} solutions were used to measure Ca^{2+} channel currents.

As a positive control for the expression of a cloned channel in tsA201 cells, pcDNA1 vectors (Invitrogen) containing rat skeletal Na^+ channel α1 cDNAs (Trimmer, *et al.*, 1989) were cotransfected into tsA201 cells with pTracer vectors. The expression of the Na^+ channel was measured in Tyrode solution by the ruptured patch clamp method. Figure 19A shows representative Na^+ channel currents from tsA cells transfected by skeletal Na^+ channel α1. Five out of 8 cells expressed 200-500 pA Na^+ currents (306 pA ± 115, mean ± S. D.). The sodium currents disappeared after switching to a 10 mM Ba^+ solution that does not contain Na^+.

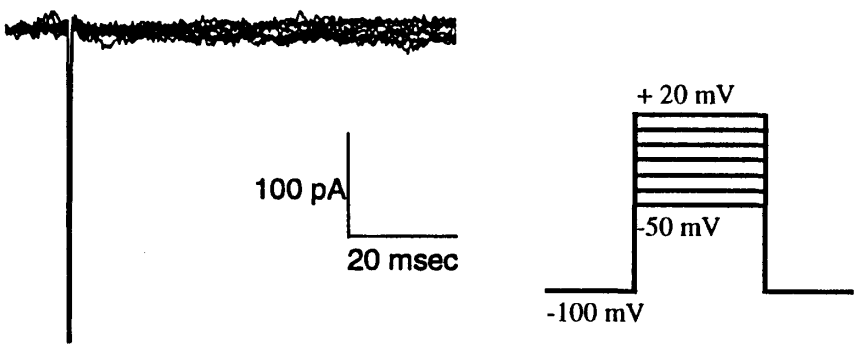
In external solutions designed to measure either Ca^{2+} or Na^+ channel currents, neither Na^+ nor Ba^{2+} currents were measured from the cells transfected with plasmids containing the Rb21-cDNA (n=43 cells out of 6 batches; figure 19B and C). These expression experiments were summarized in table 8.

In spite of extensive expression studies, currents from Rb21-channels were not expressed in either *Xenopus* oocytes or mammalian cells. Possible reasons why the channel was not expressed, and alternative methods which can be applied to express the Rb21-channel will be discussed in the next chapter.

A. Rat skeletal Na channel in Tyrode



B. Rb21-channel in Tyrode solution



C. Rb21-channel in 10 Ba solution

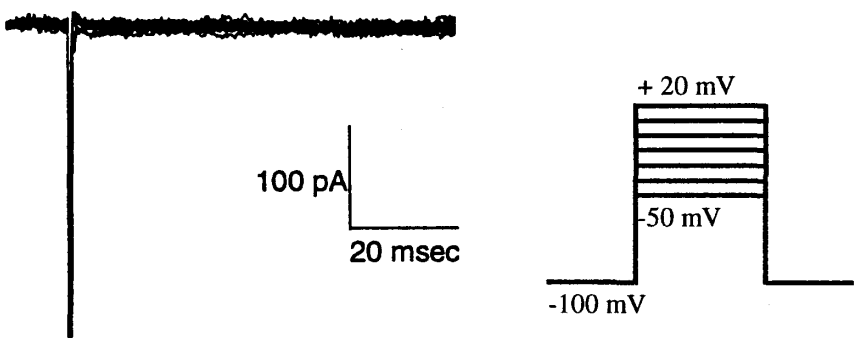


Figure 19. Electrophysiological measurements to detect the expression of the Rb21-channel in tsA 201 cells by the ruptured patch clamp method. Currents were measured from 24 to 72 hours after transfection. Currents were evoked by applying test potentials of -60 to +10 mV from a holding potential of -100 mV. As a positive control, the rat skeletal μ1 sodium channel was expressed in tsA cells. In (A), expression of the sodium channel was measured in Tyrode solution containing 140 mM Na^+. Distinctive currents were not measured from the cells transfected by plasmids containing Rb21-channel cDNA in either Tyrode solution (B) or 10 mM Ba^{2+} solution (C).

	Negative control (Blank vectors)	**Positive control** (rat μ1 Na^+ channel)	**Rb21-channel**
Number of samples	17 cells out of 4 batches	5 cells out of 8 cells	43 cells out of 6 batches
Expression	No distinct currents	200-500 pA currents	No distinct currents

Table 8. Summary of Rb21-channel expression experiments in tsA 201 cells. For the negative control, currents were measured from tsA201 cells transfected by vectors that do not contain inserts. For the positive control, currents were measured from tsA201 cells transfected by the rat skeletal muscle μ1 Na^+ channel in Tyrode solution containing 140 mM Na^+. The expression of the Rb21-channel was measured from tsA201 cells transfected by the pTracer vectors containing the Rb21-channel cDNA in both Tyrode and 10 mM Ba^{2+} solution.

CHAPTER V

DISCUSSION

5.1 Cloning of Ca^{2+} channel sequences from diverse tissues

RT-PCR cloning was extensively performed to clone a novel calcium channel. PCR primers designed from consensus sequences of reported HVA calcium channels detected six types of Ca^{2+} channel sequences from *Xenopus* oocytes, four from rat heart, five from rat brain, one from *Paramecium*, and two Ca^{2+} and one Na^{+} channel sequences from NIE-115 neuroblastoma cells.

Based on ω-conotoxin GVIA sensitivity and 9 pS single channel conductance, Lacerda and his colleagues (1994) characterized N-type and T-like currents from *Xenopus* oocytes. Dascal (1992) also characterized Ni^{2+}-sensitive currents which had a transient time course from oocytes. The properties of oocyte currents were considered to be similar to those of T-type currents. In terms of activation threshold, however, low voltage-activated Ca^{2+} channel currents were not detected at the whole cell level in either report. The single channel current amplitude of oocyte T-type channels measured during a test potential of -20 mV was significantly bigger than those of reported 7-9 pS T-type channels (Carbone and Lux, 1987; Chen and Hess, 1990). Furthermore, the properties of Ni^{2+}-sensitivity and fast inactivation overlap with the properties of high VACCs such as α1E channels (Zamponi *et*

al., 1996). In an RNA protection assay using cloned cDNA sequences from oocytes, the relative abundance of the detected calcium channels was in the following order: α1E > α1B (Cribbs, Lee, and Perez-Reyes, n=1, unpublished data). The three transcripts of α1C, α1D, and α1A were not detected. Native oocyte Ca^{2+} channel currents stimulated by α2 plus β2 contained a considerable component resistant to a mixture of 10 μM nisoldipine and 5 μM ω-conotoxin MVIIC. Nisoldipine and ω-conotoxin MVIIC were reported to block L-type channels and all of mammalian N-, P-, and Q-type Ca^{2+} channels at those doses, respectively (Randall and Tsien, 1995). The resistant component might be consistent with the dominant detection of α1E mRNA in the RNA-protection assay. Therefore, it remains to be determined whether the transient and Ni^{2+}-sensitive component might be an R-type current or a T-type current in *Xenopus* oocytes.

Four types of Ca^{2+} channel sequences were cloned from rat heart; α1C, α1D, α1A, and α1E. Except α1C sequences, detection of the other 3 sequences were not expected, because it has been an accepted dogma (Hille, 1992) that cardiac Ca^{2+} channel currents are carried through α1C-channels. A recent study showed that 30 % of cardiac Ca^{2+} channel currents were blocked by an antibody developed against an α1D sequence, indicating that α1D channels are also expressed in cardiac myocytes (Wyatt *et al.*, 1997). Tohse and his colleagues (1992) characterized a DHP-resistant Ca^{2+} channel current from ventricular myocytes of 18-day-old rat fetus. The biophysical and pharmacological characteristics of this current were different from those of T- and N-type currents. During subsequent development, the novel current becomes less prominent. Either α1A or α1E detected by PCR cloning might be a good candidate for carrying the novel current. However, the α1A

and α1E mRNA could have come from intracardiac neurons, where L-, N-, Q-, and R-type Ca^{2+} channel currents were pharmacologically identified (Jeong, 1997). Thus, the pattern of α1A and α1E expression in heart should be investigated at both the mRNA and protein level using *in situ* hybridization and immunohistochemistry.

The *Paramecium* Ca^{2+} channel homolog showed considerable conservation in S4 (voltage sensor) and pore loop regions compared to mammalian Ca^{2+} channels, while poor conservation in the other portions. The conservation of S4 and pore sequences between *Paramecium* and mammalian calcium channels implies that these portions play important functional roles in determining voltage-dependent gating and Ca^{2+} selectivity. Although the *Paramecium* channel cannot be expressed in other expression systems because ciliates use an altered codon system, the cloned channel could be expressed in *Paramecium* mutants which lack of Ca^{2+} channel currents (Caron and Meyer, 1985; Kung and Saimi, 1985). Since *Paramecium* is assumed to be one of the most primitive species expressing VACC currents (Hille, 1992), cloning and expression studies of the *Paramecium* channel might provide us important clues to understand how structure determines function in calcium channels.

Six types of high VACC sequences were amplified by primers derived from mammalian Ca^{2+} channels, demonstrating that PCR cloning is an excellent method to clone reported channel sequences. However, the designed primers did not amplify a novel calcium channel such as a T-type channel. This suggested that sequences of T-type channels are different from those of mammalian HVA Ca^{2+} channels.

5.2 Cloning of the Rb21-channel from rat brain

To clone a novel Ca^{2+} channel sequence by PCR cloning, additional sequence information of Ca^{2+} channels from diverse species may be required. Assuming that low VACCs and high VACCs diverged from an ancestral Ca^{2+} channel during evolution, another hypothesis was that low VACCs might contain conserved sequence found in Ca^{2+} channels of low-level animals. If there were sequence homology between two groups, PCR primers designed from Ca^{2+} channels of low-level species may amplify T-type channel sequences. Information of primitive Ca^{2+} channels was obtained by RT-PCR cloning and Genbank searching. A putative Ca^{2+} channel sequence of *Paramecium* was cloned by RT-PCR cloning. Other primitive Ca^{2+} channel sequences were found in the Genbank using the BLAST program (Altschul *et al*, 1990). Not only Ca^{2+} sequences of diverse species, but also a putative Ca^{2+} channel sequence of *C. elegans* (C27f2) was detected as homologous to consensus sequences in pore and S6 regions of reported Ca^{2+} channels. These results indicate that searching the Genbank is another powerful method to clone a novel gene.

A homologue (Rb21-channel) of the putative Ca^{2+} channel was cloned from rat brain by RT-PCR cloning and subsequent library screening. Structural analyses of the Rb21-channel show that the sequence is similar to reported Ca^{2+} and Na^{+} channels. Phylogenetic analysis using the Higgins and Sharp's algorithm suggested that the Rb21-channel might be an evolutionary precursor of Ca^{2+} and Na^{+} channels. However, expression of Rb21-channels in either *Xenopus* oocytes or tsA201 cells was not measured electrophysiologically, so it was not directly proven that Rb21 encodes an ion channel. Therefore, one can only speculate on physiological function of Rb21-channels, based on the literature of Ca^{2+}, Na^{+}, and cation

currents identified in brain and heart tissues.

5.3 Candidates for currents carried via Rb21-channels

The structural homology of Rb21-channel to Ca^{2+} and Na^{+} channels indicates that the putative channel is a member of the four-domain channel superfamily including voltage-activated Ca^{2+} and Na^{+} channels. Regarding the ion selectivity of Ca^{2+} and Na^{+} channels, expression studies of point mutated Ca^{2+} and Na^{+} channels in pore regions suggested that EEEE residues of Ca^{2+} channel pores determine Ca^{2+} ion selectivity, while DEKA residues of Na^{+} channels determine Na^{+} ion selectivity (Yang *et al*, 1993; Tang *et al.*, 1993; Parent and Gopalakrishnan, 1995). Compared to those residues found in Ca^{2+} and Na^{+} channels, Rb21-channel contains EEKE in the corresponding positions, suggesting that Rb21-channel might be a cation-selective ion channel, but have an ion selectivity which is distinctive from Ca^{2+} and Na^{+} channels. EEKE residues of Rb21-channel pores may form a channel that is more permeable to monovalent than divalent ions. Nevertheless, it is impossible to predict if Rb21-channel is a Ca^{2+}, a Na^{2+}, or a non-selective cation channel.

Regarding the voltage-sensitivity of channel gating, Rb21-channel may have unique gating properties different from those of reported Ca^{2+} and Na^{+} channels because the channel has fewer positive residues in S4 regions than Ca^{2+} and Na^{+} channels. The less developed S4 may cause the channel to activate and inactivate in a less voltage-sensitive manner than do known Ca^{2+} and Na^{+} channels. For example, the S4 of the Rb21-channel may cause low-threshold activation, slow activation, hyperpolarization-dependent activation, or non-inactivation. However, how the distinct S4 of the Rb21-channel will affect voltage-

sensitivity cannot be predicted.

Based on the speculation about permeation and gating of the Rb21-channel, candidate currents for the Rb21-channel were surveyed through literature. Because expression of Rb21-channel mRNA was localized in brain and heart, candidate currents were restricted to cation channel currents characterized in heart and brain tissues cells. Possible currents conducting via the Rb21-channels are as follows: (1) LVA Ca^{2+} currents, (2) non-inactivating Na^{+} currents (Taylor, 1993; Crill, 1996; Quignard *et al*, 1997), (3) hyper-polarization activated cation currents (DiFrancesco, 1993; Pape, 1996), or (4) possibly capacitative calcium currents such as I_{CRAC} activated by calcium release from internal stores (Parekh and Penner, 1997).

Three possible reasons that the Rb21-channel might carry LVA Ca^{2+} currents are the following: 1) the Rb21-channel shares structural similarity with cloned Ca^{2+} channels; 2) the distribution of Rb21-channel mRNA is consistent with the detection of LVA channel currents in brain, heart, and pancreas (Bean, 1989; Huguenard, 1996); and 3) the reversal potential of LVA channel currents is 20 - 30 mV lower than those of high VACCs, indicating that LVA channels have unique ion permeabilities different from high VACCs. These observations suggest that LVA channels may be permeable to not only Ca^{2+}, but also other monovalent ions such as Na^{+} and K^{+}. According to the previous permeation studies of the point-mutated Ca^{2+} and Na^{+} channels (Yang *et al*, 1993; Tang *et al.*, 1993; Parent and Gopalakrishnan, 1995), the presence of a positive amino acid in the domain III pore allows these channels to be permeable to monovalent ions, but not to divalent ions such as Ca^{2+} and Ba^{2+}. However, evidence suggests that additional residues in pore regions of Ca^{2+} channels

significantly affect ion selectivity (Yue and Marban, 1990; Kuo and Hess, 1993a,b). Therefore, I think that the Rb21-channel might be a good candidate for a LVA Ca^{2+} channel.

A second possibility is that Rb21-channels might carry non-inactivating Na^+ currents, which were identified in diverse neuronal cells and cardiac myocytes as well (Taylor, 1993; Crill, 1996). These sodium currents activated slowly and did not inactivate during long depolarizing test pulses. One of the reasons that the Rb21-channel may conduct Na^+ current is that the Rb21-channel shares similar structural homology with cloned Na^+ channels. The poorly developed S4 structure suggests that Rb21-channels may have slow activating and non-inactivating properties similar to those of the proposed currents. Second, expression of Rb21-channel mRNA is similarly localized to the tissues expressing these currents. Therefore, the Rb21-channel might be a candidate for conducting non-inactivating Na^+ currents in neuronal cells and cardiac myocytes.

A third possibility is that Rb21-channels might carry hyperpolarization-activated cation currents, which were characterized from brain and heart cells (DiFrancesco, 1993; Pape, 1996). The currents carried by Na^+ and K^+ play important roles in pacemaking activity of action potentials in those cells. One of the reasons that the Rb21-channel is a candidate for conducting a hyperpolarization-activated cation current is that the positively-charged residues of Rb21-channel pores may make the channel more permeable to monovalent than divalent ions as discussed above. A second reason is that hyperpolarization-activated channels are similarly localized to Rb21 mRNA distribution. Therefore, the Rb21-channel may be a candidate for conducting these cation currents.

Additionally, the Rb21-channel might carry I_{CRAC} currents, which are activated by

calcium release from internal stores (Parekh and Penner, 1997). In terms of Ca^{2+} selectivity, the pore of the I_{CRAC} channel might be similar to those of cloned VACC channels, because the I_{CRAC} channel is selectively permeable to Ca^{2+}. The structural similarity of the Rb21-channel and cloned Ca^{2+} channels shown in figure 14 supports the hypothesis that the Rb21-channel might carry I_{CRAC} currents.

Although it was proposed that the Rb21-channel might conduct LVA Ca^{2+} currents, non-inactivating Na^{+} currents, hyper-polarization activated cation currents, or I_{CRAC}, it can not be ruled out that the Rb21-channel may conduct an unexpected type of current.

5.4 Experimental approaches to identify the function of Rb21-channels

One of possible reasons that I was not able to measure Rb21-channel currents in oocytes and HEK cells is that those expression systems might not properly process either the transcription, translation, or post-transcription modification of the channel, nor express it on the plasma membrane. Reviewing the history of cloned Ca^{2+} channel expression, skeletal L-type $\alpha 1$ subunit was expressed in dysgenic myotubules and mouse L-cells, but not in *Xenopus* oocytes (Perez-Reyes and Schneider, 1994). Recently, the skeletal $\alpha 1$ subunit was expressed in *Xenopus* oocytes with coexpression of $\beta 1b$ subunit, suggesting that $\beta 1b$ may be a necessary element to express the skeletal $\alpha 1$ subunit in oocytes (Ren and Hall, 1997). Antisense depletion of endogenous β subunits in oocytes inhibited expression of human $\alpha 1E$ subunits (Tareilus *et al.*, 1997). This observation provides another example that expression of $\alpha 1E$ in *Xenopus* oocytes requires the presence of endogenous β subunits. Similarly, Rb21-channel expression may require the presence of auxiliary subunits. Therefore, expression of

the Rb21-channel should be studied in other expression system such as CHO (Chinese hamster ovary), COS (African green monkey kidney), and Sf9 cells (*Spodoptera frugiperda*) (Chaput *et al*, 1988; Gluzman, 1981; Smith *et al.*, 1985).

If the Rb21-channel is not expressed in other systems, an indirect approach can be attempted via expression studies of chimeras constructed between the Rb21-channel and either a cloned Ca^{2+} or Na^{+} channel. Because the Rb21-channel shares similar structural features with cloned Ca^{2+} or Na^{+} channels, chimeras constructed between Rb21-channel and a cloned Ca^{2+} channel might be expressed in oocytes or HEK 293 cells. If some chimeras are expressed and measured as currents, biophysical characterization of expressed currents might give clues indicating what type of current can be conducted by the Rb21-channel. Based on this hypothesis, I have constructed a chimera with the 5'-end initiation portion before domain ISI of Rb21-channel replaced with the corresponding portion of human α1E (Schneider *et al*, 1995). Unfortunately, no currents could be recorded in oocytes injected with cRNA from this construct. Nevertheless, this approach utilizing chimera construction and expression might be helpful to express not only Rb21-channel but also the following putative channels that have not been expressed yet: a Na^{+} channel from a jellyfish (Anderson *et al.*, 1993); atypical Na^{+} channels from mouse and human (Nav 2.1 and Nav2.2;) George *et al*, 1992; Felipe *et al.*, 1994); a Na^{+} channel from squid (Sato and Matsumoto, 1992); and a Ca^{2+} channel from fruitfly (Zheng *et al.*, 1995).

The third possible approach to investigate the identity of the Rb21-channel is based on the combined experiments of localization, biophysical identification, and antisense oligonucleotide depletion of the Rb21-channel. After identifying where the Rb21-channel

mRNA is expressed using *in situ* hybridization, the electrophysiological properties of the channel can be identified by comparing controls to cells treated with antisense-oligonucleotides. Similar experiments can also be performed at the protein level using antibodies developed against the Rb21-channel. Distribution of the Rb21-channel protein could be localized using labeled antibodies specific to the Rb21-channel. For example, if the Rb21-channel is localized to the endoplasmic reticulum like the IP_3 receptor, the location of the channel could be found by immunohistochemistry using antibodies specific to the Rb21-channel (Mignery *et al.*, 1989). Antibodies developed against external sequences of Rb21-channel pores may directly block the currents carried by the Rb21-channels, allowing the identification of currents conducted by the Rb21-channel.

Similarly, the physiological function of the Rb21-channel could be identified by knocking out the gene of Rb21-channel in mouse, or the corresponding gene in less complex species such as *C. elegans* and *Drosophila* (Wickman *et al.*, 1998). Comparing physiological properties between a normal group and a gene-depleted group may provide an answer to the function of Rb21-channels.

The fourth approach would involve protein studies. The reasons why the Rb21-channel was not expressed as a membrane ion channel could be investigated by following the translation and post-translation processing of the Rb21-channel in expression systems, such as *Xenopus* oocytes and HEK293 cells. First of all, whether or not the transcript of the Rb21-channel is transcribed into protein should be investigated. If the transcripts are not translated into an expected size protein, why the transcripts are not translated should be studied. If the transcript is translated into the expected size of protein, which could be

detected by Western blotting of SDS-PAGE gels, the post-translation processes including glycosylation and targeting should be assessed. To solve these difficulties, a similar method using chimeras explained above could be applied.

CHAPTER VI

SUMMARY AND CONCLUSIONS

6.1 Summary

Calcium influx through voltage-activated Ca^{2+} channels (VACCs) plays essential roles in various physiological functions such as: membrane depolarization, muscle contraction, hormone secretion, synaptic transmission, cell viability, and gene expression. Electrophysiological and pharmacological studies have identified how Ca^{2+} channels are related to pivotal physiological functions. Classification of VACCs has been established on the basis of biophysical and pharmacological criteria. Basically, VACCs can be classified into low VACCs having a low voltage threshold of activation and high VACCs having a high voltage threshold of activation. Molecular cloning and expression studies of Ca^{2+} channel $\alpha 1$ subunits have uncovered new aspects about structure, diversity, pharmacology, and biophysical properties of Ca^{2+} channels. According to expression studies on cloned Ca^{2+} channel $\alpha 1$ subunits, high VACCs can be classified as follows: $\alpha 1S$ is a skeletal L-; $\alpha 1C$ is a cardiac L-; $\alpha 1D$ is a neuro-endocrine L-; $\alpha 1A$ is a P/Q-; $\alpha 1B$ is a N-; and $\alpha 1E$ is a R-type Ca^{2+} channel $\alpha 1$ subunit. However, there still remain Ca^{2+} channels, such as T-type channels, which have not been cloned.

To clone a novel Ca^{2+} channel, reverse transcription-polymerase chain reaction (RT-

PCR) cloning methods were applied. The initial hypothesis was that novel Ca^{2+} channels contain consensus amino acid sequences found in mammalian Ca^{2+} channels. PCR primers designed from conserved amino acids in Ca^{2+} channel $\alpha 1$ subunits might amplify not only already cloned $\alpha 1$ sequences, but also novel $\alpha 1$ sequences such as those of T-type channels. PCR substrates (cDNAs) were reverse-transcribed from mRNAs isolated from various tissues and cell lines that have been reported to express T-type Ca^{2+} channel currents. PCR products were separated, isolated, cloned, and sequenced.

Deduced amino acid sequences were compared to those of reported Ca^{2+} channel sequences to identify PCR products. The comparison showed that a pair of PCR primers derived from mammalian Ca^{2+} channels amplified a putative Ca^{2+} channel cDNA fragment from *Paramecium tetraurelia.* The sequence cloned from *Paramecium* showed 29-41 % sequence homology with those of reported Ca^{2+} channels. However, primers designed on the basis of mammalian Ca^{2+} channels amplified only reported Ca^{2+} channel sequences from tissues expressing T-type Ca^{2+} channel currents.

Another strategy to design PCR primers was based on conserved amino acid sequences found in less complex animal species. I assumed that low VACCs and high VACCs might be diverged from an ancestral Ca^{2+} channel during evolution and found in low-level, living organisms. We searched for a Ca^{2+} channel-like sequence in the Genbank and found a candidate in a *Caenorhabditis elegans* cosmid (C27f2). The deduced amino acid sequence showed homology to reported Ca^{2+} channels. Furthermore, the whole structure of the putative Ca^{2+} channel had four repeated domains, each of which contains 6 putative membrane spanning segments and a pore loop. To clone a rat version of the putative Ca^{2+}

channel found in *C. elegans*, several PCR primers were designed based on conserved amino acid sequences. One pair of PCR primers amplified PCR products from the rat brain. The translated sequence of the clone designated Rb21 was homologous to reported Ca^{2+} channel sequences, but could not be classified into any group of established Ca^{2+} or Na^{+} channels. This analysis suggested that Rb21 was a partial cDNA sequence of a putative novel ion channel resembling Ca^{2+} and Na^{+} channels. The full length cDNA of the Rb21-channel was cloned by screening cDNA libraries of the rat brain. The cloned cDNA of the Rb21-channel contains 6820 bases. The open reading frame is 5,214 bases and the molecular weight of the deduced amino acid sequence is about 205 kdaltons. The Rb21-channel shares considerable amino acid sequence homology with Ca^{2+} and Na^{+} channels. Sequence alignment, hydropathicity analysis, and matrix homology plots suggest that the Rb21-channel has a four repeat structure and each repeat has six membrane spanning regions similar to Ca^{2+} and Na^{+} channels. According to phylogenetic relationships, Rb21-channel might be an ancestral type of Ca^{2+} and Na^{+} channels. Northern blot analyses showed that the size of detected transcripts is about 6.9 kb and the transcripts of the Rb 21-channel are expressed predominantly in the brain, moderately in the heart, and weakly in the pancreas. Despite extensive expression studies, the expression of the Rb21-channel was not detected as currents in *Xenopus* oocytes and HEK-293 cells. Based on their location and structural characteristics, I propose that the Rb21-channel might be either a LVA Ca^{2+} channel, a non-inactivating Na^{+} channel, or a hyperpolarization-activated cation channel.

6.2 Conclusions

A putative ion channel (the Rb21-channel) was cloned using a combination of Genbank search, RT-PCR, and library screening. Structural analyses of the Rb21-channel suggested that the putative channel shares similar characteristics with voltage-activated Ca^{2+} and Na^{+} channels. From phylogenetic relationships, the Rb21-channel was predicted to be a primordial channel to Ca^{2+} and Na^{+} channels. Based on the sequence and structural distinctiveness, I propose that the Rb21-channel might be a voltage-activated cation channel with unique voltage gating properties.

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VITA

Jung-Ha Lee, the son of Jae-Yeong Lee and Jong-Ok Lee, was born in Samchuck, on the east coast of Republic of Korea on May 20, 1961. He attended Sogang University in Seoul in February 1980, and graduated in February, 1984 with the bachelor degree of Science in Biology. His graduate study was continued at the same institute. He received a Master Degree of Science in August, 1986. After serving a military obligation for 4 years, he worked as a lecturer at Sogang university.

He entered the Ph.D. program in the Department of Physiology at Loyola University of Chicago in January, 1993. His dissertation work was completed under the direction of Dr. Edward Perez-Reyes. He is a member of the Korean Physiology Society and the Biophysical Society. He married Soo-Kyung Kim, who unfortunately passed away during his dissertation work, and has a daughter, Marie Lee.

PUBLICATIONS

A. Journals

1. Bernal, J., **Lee, J.-H.**, Cribbs, L. L., and Perez-Reyes, E. (1997): Full reversal of Pb^{2+} block of L-type calcium channels requires treatment with heavy metal antidotes. *J. Pharmacol. Exp. Ther.* 282: 172-180.

2. Perez-Reyes, E., Cribbs, L. L., Daud, A., Lacerda, A. E., Barclay, J., Williamson, M. P., Fox, M., Rees, M., and **Lee, J.-H.** (1998): Molecular characterization of a neuronal low voltage-activated T-type calcium channel. *Nature* 391:896-900.

3. Cribbs, L. L., **Lee, J.-H.**, Satin, J., Zhang, Y., Daud, A., Barclay, J., Williamson, M. P., Fox, M., Rees, M., and Perez-Reyes, E. (1998): Cloning and characterization of α1H from human heart, a member of the T-type calcium channel gene family. *Circ. Res.* (Accepted).

4. **Lee, J.-H.**, Cribbs L. L., Daud, A., and Perez-Reyes E. (1998): Permeation properties of a neuronal T-type calcium channel (α1G; CavT.1). (in preparation)

5. **Lee, J.-H.**, Cribbs L. L., and Perez-Reyes E. (1998): Molecular and pharmacological characterization of endogenous *Xenopus* oocyte calcium channels. (in preparation).

6. **Lee, J.-H.**, Cribbs L. L., and Perez-Reyes E. (1998): Cloning and expression of a novel ion channel resembling calcium and sodium channels. (in preparation).

B. Abstract

1. Bernal, J., Ruvalcaba, S., **Lee, J.-H.**, and Perez-Reyes, E. (1995): The cardiac L-type calcium channel is modulated by lead. *Biophys. J.* 68: A108.

2. Bernal, J., **Lee, J.-H.**, and Perez-Reyes, E. (1996): Lead is a potent blocker of cardiac L-type calcium channels. *Biophys. J.* 70: A186.

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4. Perez-Reyes, E., **Lee, J.-H.**, Daud, A., and Cribbs, L. L. (1997): Cloning of calcium channels representing two new subfamilies distinct from high voltage-activated types. *Biophys. J.* 72: A245.

5. Cribbs, L. L., Yang, J., **Lee, J.-H.**, Daus, A., and Perez-Reyes, E. (1998): Molecular cloning of a low voltage-activated calcium channel from rat brain. *Biophys. J.* 74:A119.

6. Perez-Reyes, E., Cribbs, L. L., Daus, A., Lacerda, A. E., and **Lee, J.-H.** (1998): Biophysical characterization of the first cloned T-type calcium channel. *Biophys. J.* 74:A119.

7. **Lee, J.-H.**, Cribbs, L. L., and Perez-Reyes, E. (1998): Cloning of a novel ion channel: A possible evolutionary precursor to calcium and sodium channel. *Biophys. J.* 74:A316.

DISSERTATION APPROVAL SHEET

The dissertation submitted by Jung-Ha Lee has been read and approved by the following committee:

Edward Perez-Reyes, Ph.D. (Director)
Assistant Professor
Department of Physiology
Loyola University Chicago

Stephen B. Jones, Ph.D.
Associate Professor
Department of Physiology
Loyola University Chicago

Gregory A. Mignery, Ph.D.
Assistant Professor
Department of Physiology
Loyola University Chicago

Robert R. Wurster, Ph.D.
Professor
Department of Physiology
Loyola University Chicago

Roman Shirokov, Ph.D.
Assistant Professor
Department of Molecular Biophysics & Physiology
Rush Medical Center

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosopy.

April 1, 1998
Date

Director's Signature